

# **DEVELOPMENT OF AN *IN VITRO* MODEL TO INVESTIGATE REPEAT OCULAR EXPOSURE**

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# Abstract

The Draize eye irritation test has been widely adopted as the “gold standard” to evaluate the potential eye irritation of a wide range of chemicals and formulations, including; pharmaceuticals, cosmetics, and their raw ingredients. The rationale for pursuing the development of human ocular based *in vitro* alternatives is to provide greater confidence in the prediction of human reactions to mild and moderate chemicals. This is particularly important with the implementation of the EC White Paper, "Strategy for a future chemicals policy" (2001) that is estimated to require the testing of approximately 30,000 'existing' chemicals by 2012.

The development of *in vitro* alternatives for toxicity testing has mainly focused upon tests for quantitative measurement of acute toxicity following a single high-dose exposure. However, the degree of toxicity of any exposure is a function of; the dose which target cells receive, the duration of the exposure and the ability of the exposed cells to recover from the exposure. However, little account is taken of the potential role of long-term effects in modulating the toxic response. This study aims to generate an *in vitro* model utilizing a human corneal cell line monolayer to investigate the effects chronic exposure to exogenous chemicals has upon toxicity of a subsequent acute challenge.

Surfactants are ubiquitous within our daily environment, being significant active components in both household and personal care products, cosmetics and pharmaceuticals. Initially the effects of four representative surfactants (sodium dodecyl sulphate; anionic, tween 20; non-ionic, cocamidopropylbetaine; amphoteric and benzalkonium chloride; cationic) were examined following chronic exposure.



Although the measured endpoints (neutral red uptake, resazurin reduction, fluorescein leakage and total protein content) revealed no alterations in J-HCET morphology, barrier function or biochemistry as a consequence of chronic exposure, it was determined that pre-exposure modulated the toxicity of subsequent acute exposures. The observed modulation in toxicity could have significant health implications for personal care products, cosmetics and pharmaceuticals intended for use near or within the eye. However, the mechanism(s) by which the toxicity of subsequent surfactant exposures was modulated remains to be elucidated.

Whilst standard surfactants are good indicators of the effects following chronic exposure, there are pharmaceuticals designed for repeat use in the eye that have been associated with long term ocular irritancy, and a discontinuation of use i.e. timolol maleate. J-HCET cultures exposed to BSO *in vitro* confirm that the toxicity of timolol to the human corneal cells line was enhanced by suppressing the activity of  $\gamma$ -glutamylcysteine synthetase through irreversible inhibition, resulting in a decrease in intracellular glutathione (GSH) levels. In addition, chronic exposures to timolol maleate were also associated with an increase in toxicity of subsequent acute challenges. The long term use of Timolol maleate in the treatment of glaucoma *in vivo* may result in similar alterations in the intracellular GSH concentrations, resulting in discontinuation of treatment as consequence of ocular irritation through the generation of reactive oxidation species beyond the threshold that depleted intracellular GSH can respond.

Since *in vitro* methods have been, and are being developed as alternatives to animal experiments, the use of bovine serum as a source of growth factors can seem to be contradictory to the purposes of the Three R's concept of Russell and Burch (1959).

This study was conducted using culture media that contain animal derived growth supplements and a media where these had been substituted for plant derived materials. Comparisons were made between the effects these two supplements had upon a number of biological factors including morphology, biochemistry, barrier function and the response to exogenous chemicals. No alterations were observed in these parameters as a consequence of using culture medium containing plant derived materials compared to those containing animal derived growth supplements.

This study has demonstrated that the development of a reliable and reproducible *in vitro* assay in keeping with the principles of the Three R's for modeling chronic - repeat ocular irritation is possible. However, the mechanistic relevance of the endpoints chosen and cell layer ultrastructure is considered to be an essential component. The further development of cell based *in vitro* systems to predict human responses to chronic/repeat ocular irritation is required.

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# Abbreviation List

<b>3D</b>	:	Three dimensional
<b>7H6</b>	:	7H6 antigen
<b>AF</b>	:	Actin filament
<b>AJ</b>	:	Adherens junction
<b>BAK</b>	:	Benzalkonium chloride
<b>BOC</b>	:	British oxygen company
<b>BPE</b>	:	Bovine pituitary extract
<b>BSA</b>	:	Bovine serum albumin
<b>BSO</b>	:	Buthionine sulphoxamine
<b>c-AMP</b>	:	Cyclic adenosine monophosphate
<b>CAM-TB</b>	:	Chorioallantoic membrane-trypan blue staining method
<b>CAPB</b>	:	Cocamidopropylbetaine
<b>CF</b>	:	Corneal fibroblasts
<b>CHL-CVS</b>	:	Chinese hamster lung using crystal violet staining method
<b>CIG</b>	:	Cold insoluble globulin
<b>Cld</b>	:	Claudin
<b>CMC</b>	:	Critical micelle concentration
<b>CO<sub>2</sub></b>	:	Carbon dioxide
<b>COLIPA</b>	:	Cosmetic toiletry and perfumery association
<b>dH<sub>2</sub>O</b>	:	Distilled H <sub>2</sub> O
<b>DMSO</b>	:	Dimethylsulphoxide
<b>DNA</b>	:	Deoxyribonucleic acid
<b>DP</b>	:	Desmoplakin
<b>Dsc</b>	:	Desmocollin
<b>Dsg</b>	:	Desmoglein
<b>E-Cadherin</b>	:	Epithelial cadherin
<b>ECETOC</b>	:	European centre for the ecotoxicology and toxicology of chemicals
<b>ECVAM</b>	:	European centre for the validation of alternative methods
<b>EDTA</b>	:	Ethylenediaminetetraacetic acid
<b>ELISA</b>	:	Enzyme linked immunosorbant assay
<b>EU</b>	:	European union
<b>f-actin</b>	:	Fibrous actin
<b>FBS</b>	:	Foetal bovine serum
<b>FCS</b>	:	Foetal bovine serum
<b>FITC</b>	:	Fluorescein isothiocyanate
<b>FLA</b>	:	Fluorescein leakage assay
<b>FNC</b>	:	Fibronectin-collagen
<b>FRAME</b>	:	Fund for the replacement of alternative methods
<b>GSH</b>	:	Glutathione
<b>GSSH</b>	:	Glutathione disulphide
<b>HBSS</b>	:	Hanks balanced salt solution
<b>HCE-T</b>	:	Gillette human corneal epithelial cells
<b>HD</b>	:	Haemoglobin denaturation method
<b>HeLa-</b>	:	Cervical epitheliod (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
<b>MTT</b>	:	bromide))
<b>HET-CAM</b>	:	Hens egg chorioallantoic method
<b>HIV</b>	:	Human immunodeficiency virus
<b>HKGS</b>	:	Human keratinocyte growth supplement
<b>HKGS-V2</b>	:	Human keratinocyte growth supplement version 2
<b>HO</b>	:	Home Office
<b>IACUC</b>	:	Institutional animal care and use committee
<b>IBM</b>	:	Industrial business machines

<b>ICCVAM</b>	:	Interagency co-ordinating committee on the validation of alternative methods
<b>INVITTOX</b>	:	<i>In vitro</i> techniques in toxicology
<b>J-HCET</b>	:	Japanese human corneal cells
<b>KB</b>	:	Kenacid blue
<b>kDa</b>	:	Kilodalton
<b>KF</b>	:	Keratin filament
<b>Kv</b>	:	Kilovolts
<b>LDM</b>	:	Living dermal model
<b>MDCK</b>	:	Madin Derby canine kidney
<b>MF</b>	:	Myofibroblast
<b>MLC</b>	:	Myosin light chain
<b>MLCK</b>	:	Myosin light chain kinase
<b>MMAS</b>	:	Modified maximum average score
<b>MTT</b>	:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
<b>NGS</b>	:	Normal goat serum
<b>NRR</b>	:	Neutral red release
<b>NRU</b>	:	Neutral red uptake
<b>Oc</b>	:	Occludin
<b>OECD</b>	:	Organisation for economic co-operation and development
<b>PBS</b>	:	Phosphate buffered saline
<b>PC</b>	:	Personal computer
<b>PG</b>	:	Plakoglobin
<b>Plc</b>	:	Public limited company
<b>R/SF</b>	:	Resazurin-sodium fluorescein
<b>RBC</b>	:	Red blood cell haemolysis method
<b>REACH</b>	:	Registration, evaluation and authorization of chemicals
<b>Res</b>	:	Resazurin reduction
<b>RIA</b>	:	Radiological immunoassay
<b>ROS</b>	:	Reactive oxygen species
<b>RT-PCR</b>	:	Reverse transcriptase polymerase chain reaction
<b>SD</b>	:	Standard deviation
<b>SDS</b>	:	Sodium dodecyl sulphate
<b>SIRC</b>	:	Statens Seruminstitut rabbit corneal cells
<b>SIRC-CVS</b>	:	Statens Seruminstitut rabbit corneal cells using crystal violet method
<b>SIRC-NRU</b>	:	Statens Seruminstitut rabbit corneal cells using neutral red uptake
<b>SOD</b>	:	Superoxide dismutase
<b>SV40</b>	:	Simian virus 40
<b>T20</b>	:	Tween 20
<b>Tag</b>	:	T antigen
<b>TCPS</b>	:	Tissue culture plasticware
<b>TEA</b>	:	Tissue equivalent assay
<b>TEM</b>	:	Transmission electron microscopy
<b>TER</b>	:	Transepithelial resistance
<b>TIC</b>	:	Type I collagen
<b>TJ</b>	:	Tight junction
<b>TRX</b>	:	Thioredoxin
<b>Vn</b>	:	Vitronectin
<b>ZO-1</b>	:	Zonula occludens-1
<b>ZO-2</b>	:	Zonula occludens-2
<b>ZO-3</b>	:	Zonula occludens-3

# **Chapter 1**

## ***Introduction***

# Introduction

## 1.1 The Scientific Procedures Act (1986) and acute toxicity testing

The need for alternatives to animal experimentation is increasingly recognised particularly with the advent of the registration, evaluation and authorization of chemicals (REACH) programme (Worth and Balls, 2002a), even though total eradication of animal use is not possible at the moment, for example if valuable medical research is to continue. The use of animals in scientific procedures is regulated by the Animals (Scientific Procedures) Act 1986, which is widely viewed as the most rigorous piece of legislation of its type in the world (Van der Valk *et al.*, 2004). It acknowledges the requirements of European Union Directive 86/609/EEC and offers a high level of protection to animals whilst recognising the need to use animals in determining chemical safety, human risk assessment, medical research, the development of new medicines and “blue skies” scientific research. The Act regulates scientific procedures which may cause pain, suffering, distress or lasting harm to “protected animals”; it refers to these as “regulated procedures”. “Protected animals” are defined in the Act as all living vertebrate animals, except humans, as well as one invertebrate species, the common octopus. This Act recognises that scientific procedures can only be permitted when the benefits that the work is likely to bring (to humans, other animals or the environment) outweigh any pain or distress that the animals may experience. The Animal (Scientific Procedures) Act 1986, which was a total revision of the 1886 act and was itself amended in 1988, requires any persons planning to undertake animal testing to consider alternatives prior to beginning their research. Researchers must carry out a thorough search of the



relevant literature and report both their findings and the databases used for these searches to the Institutional Animal Care and Use Committee (IACUC). If no alternatives are available, a personal and project license application must be made to the Animal Procedures Committee before animal work will be considered. The 1986 Act has a three-level licensing system; those carrying out procedures must hold personal licences, the programme of work must be authorised in a project licence, and work must also normally take place at a designated user establishment. In addition to controlling scientific procedures the Act also covers the breeding of animals with harmful genetic defects, the breeding of genetically manipulated animals, the production of antisera and other blood products and the maintenance of tumours and parasites. Of the animals bred for experimental research, one of the major consumers is that of the chemical and pharmaceutical industries for toxicological studies. The assessment of the *in vivo* toxicity of products and their constituents has conventionally been based on four elements; hazard identification, hazard characterisation, exposure assessment and risk characterisation.

### **Hazard identification**

The identification of the inherent capacity of a chemical to cause one or more adverse effects, without regard to the likelihood or severity of such effects.

### **Hazard characterisation**

The (semi-) quantitative evaluation of the nature of the adverse effects following the exposure to a chemical, including the assessment of toxic potency (the relative toxicity of a chemical) and, where possible, a dose response assessment.

## **Exposure assessment**

The (semi-) quantitative evaluation of the likely exposure of humans and/or the environment to a chemical.

## **Risk characterisation**

The (semi-) quantitative estimation of the probability that an adverse effect will occur, and of its severity and duration in a given population under defined exposure conditions, based on the previous three elements.

The various elements of the risk assessment paradigm encompass a variety of experimental activities, animal experimentation, chemical analysis, biomonitoring on animals or humans and computer modelling. Animal based studies generally lead to observations on the clinical, histological and/or functional changes in the animals caused by a single or repeat exposure to a defined dose of the chemical under study. The adverse effects can be defined as a change in the morphology, physiology, growth, development or life-span of an organism that results in an impairment of its functional capacity or ability to compensate for additional stress, or an increased susceptibility to the harmful effects of other environmental influences (IPCS, 1978). Acute local toxicity studies are performed to determine the local toxic effects that may result from a single exposure to a chemical or product, by way of the oral, dermal, ocular or inhalation routes. The exposure may be incidental, accidental or deliberate (for example, in the case of pharmaceuticals and certain cosmetics).

Of the toxic effects this project will concentrate on those related to ocular irritation that can result in corrosion. Essentially four different approaches to the evaluation of ocular hazard have been adopted, some of which have been used in the international regulation of chemical products Kay and Calandra (1962), FHSA (1974), NAS (1977), WHMIS (1987), EEC (1992). The classification procedures are similar since they all are based on the Draize rabbit eye model and utilize the Draize scoring system for ranking ocular responses.

## **1.2 The Draize eye irritation test (determination of ocular irritancy *in vivo*)**

Most people do not consider the vulnerability of their eyes. Loss of eyesight can happen at work or at home by accidental exposure to products ranging from manufacturing solvents to residential cleaners. Yet few people think about how the use or misuse of preparations could have life changing effects (Wilhelmus, 2001). Ocular toxicology testing developed during the 20<sup>th</sup> century as the pharmaceutical industry grew (Green, 1992) and as their products were found to have effects upon the eye. The Draize eye irritation test (Draize test) (Draize *et al.*, 1944) has been widely adopted as the “gold standard” to evaluate the potential eye irritation of a wide range of chemicals and formulations including pharmaceuticals, cosmetics, and their raw ingredients. It was originally conceived to assess the safety of products intended to go onto skin (Draize *et al.*, 1944). The Draize test has been applied conventionally to investigate the safety of cosmetic and pharmaceutical products and their ingredients because it directly represents eye irritation due to chemical insult. The assay generates a score that is based on observations of irritant response by the

cornea, conjunctiva, iris and/or nictating membrane after exposure to test compounds, including an evaluation or reversibility of injury (table 1.01).

**Table 1.01** : The Draize eye irritation assay scoring system (maximum of 110) for ranking responses.

Ocular Structure	Observation	Score
Cornea	A No ulceration or opacity	0
	Scattered or diffuse area of opacity	1
	Easily discernible translucent area, details of iris slightly obscured	2
	Necrotic area, no details of iris visible, size of pupil barely discernible	3
	Opaque cornea, iris not discernible through the opacity	4
	B One Quarter (or less) but not zero	1
	Greater than one quarter, less than one half	2
	Greater than one half, less than three quarters	3
	Greater than three quarters, upto whole area	4
	Score = A X B X 5 (range, 0 to 80)	
Iris	A Normal	0
	Iris still reacting to light (sluggish reaction is positive)	1
	No reaction to light, haemorrhage, gross destruction (any or all of these)	2
	Score = A X 5 (range, 0 to 10)	
Conjunctivae	A Blood vessels normal	0
	Some blood vessels definitely hyperaemic (injected)	1
	Diffuse, crimson colour, individual vessels not easily discernible	2
	Diffuse beefy red	3
	B Any swelling above normal	1
	Obvious swelling with partial eversion of the lids	2
	Swelling with lids about half closed	3
	Swelling with lids about half closed to completely closed	4
	C Discharge above normal	1
	Discharge with moistening of the lids and hairs just adjacent to the lids	2
	Discharge with moistening of the lids and considerable area around the eye	3
	Score = (A + B + C) X 2 (range, 0 to 20)	
Lids and/or nictating membranes	No swelling	0
	Any swelling above normal (includes nictating membranes)	1
	Obvious swelling with partial eversion of lids	2
	Swelling with lids about half closed	3
	Swelling with lids more than half closed	4

The separate scores for each ocular tissue are then weighted, combined and the maximum of these average scores is then taken as the modified maximum average score (MMAS) which is then used to determine the irritancy potential (table 1.02).

For most purposes the MMAS scores between 15 and 50 are the most clinically relevant.

**Table 1.02 :** Classification of chemicals according to the Draize rabbit eye test (adapted from Vian *et al.*, 1995)

MMAS	Irritant Potential
≤ 15	Mild irritant
> 15 to ≤ 30	Moderate irritant
> 30 to ≤ 50	Irritant
> 50 to ≤ 110	Corrosive

Although an organisation for economic co-operation and development (OECD) guideline exists for the Draize eye irritation assay. The specific protocols for testing the ocular toxicity of compounds vary among countries. Testers must ensure that flaws in the conduct and analysis are minimal, that sources of bias have been considered and controlled, and that adequate precision have been assured by study design (Wilhelmus, 2001). Typical components of Draize eye irritation testing are listed in table 1.03. Key parameters are; the type and number of model system, the dose and duration of exposure, and the spectrum and timing of outcome measures.

**Table 1.03 : Test parameters of the Draize eye irritation assay (adapted from Wilhelmus, 2001)**

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Albino rabbits (e.g., New Zealand white rabbits) are the usual test species; animals should be acclimated to the test environment. Animals are evaluated before exposure to ensure normal eyes; cages should be designed to prevent accidental injury. 3 to 6 animals are generally used per test dose, although a single animal may serve as a sentinel before further testing. One eye (e.g., right eye) is designated to the test; the contralateral eye serves as a matched control and is used for baseline evaluation. A dose volume of approximately 100µl per eye was formally used, but low-volume doses (e.g., 10µl) may be used. Topical anaesthetics are not usually recommended. Instillation is made into the lower conjunctiva cul-de-sac; blinking is allowed although the eyelids may be held open. Sterile saline or balanced salt solution may be used to irrigate the ocular surface. Pre- and post-exposure evaluation is performed by external observation under proper illumination. Evaluations are generally made at 1, 24, 48 and 72 hours after exposure, and if necessary at 7 and 21 days. Ocular changes are graded by a scoring system that includes rating any alterations to the eyelids, conjunctiva, cornea and iris.

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The evaluation of ocular lesions is subjective and susceptible to variability between observers and laboratories (Wilhelmus, 2001). The focus of eye irritation testing is on assessing observable mucosal and epithelial adverse effects. Systemic toxicity is not usually an outcome measure. However, any nonocular reaction resulting from drug absorption by the blood vessels and lymphatics of the conjunctiva or nasolachrymal outflow system should be noted. Even with this safeguard, there remains a considerable risk of overestimation of hazard when utilising the Draize test, and this was demonstrated by the early work of Beckley (Beckley, 1965; Beckley *et al.*, 1969). They observed that when performed with a liquid detergent, the effects of 100µl of the undiluted product in rabbits and humans, the effects in rabbit were significantly greater than seen in human volunteers, suggesting that the rabbit Draize test does not reflect the eye irritation in humans (Roggeband *et al.*, 2000). There is an increasing realisation of the limitations of animal models in relation to human ocular toxicology, as fundamental differences between species are identified (Wilson, 2000; Putnman *et al.*, 2002). For example, the rabbit possesses a

nictitating membrane, the primary role of which is to protect the eye against injury and further moisturize the cornea (Chan and Hayes, 1994) essentially absent in humans. In addition the rabbit demonstrates a thinner cornea, a less effective tear film, looser palpebrae, slower blink reflex and a higher pH (8.2 - 8.5) (Chan and Hayes, 1994; Kajima *et al.*, 1995; Sina *et al.*, 1995). All of which contribute to a generalised increased sensitivity to chemical insult (Swanston, 1993; Chan and Hayes, 1994) in the rabbit eye. It can be argued that over sensitivity increases the safety margin, and hence is a desirable feature of the *in vivo* test.

### **1.3 Limitations of the Draize test for predicting irritation potential**

Social and scientific criticism (table 1.04) of the Draize test and the reticence to test directly on humans, has stimulated researchers to develop *in vitro* alternatives that provide a quantitative and mechanistic evaluation of cellular damage. Given the complexity of the mechanisms involved in ocular response to irritants, it is especially challenging to develop *in vitro* models that are predictive of ocular irritation *in vivo*, and meet the validation criteria for *in vitro* assays (Brantom *et al.*, 1997).

**Table 1.04** Benefits and limitations of the Draize eye irritation assay (adapted from Wilhelmus, 2001).

Criteria	Advantages	Drawbacks
Training	Ease of learning and performing the test	Variable consistency among multiple examiners
Exposure	Allows complex mixtures and specific ingredients to be tested at various concentrations	Reflex blinking, tearing, and other responses may affect results
Severity rating	Aids in comparing relative effects of different compounds	Ordered effects are not linear; range of scores is not uniform for different ocular tissues
Observations	Offers ability to observe healing and recovery	Scored components are often limited to visible conjunctival, corneal, and iris changes
Scoring	Provides overview of the onset, duration, severity, and resolution of adverse effects of a substance on the eye	Weighting of test components is arbitrary; summary score mask tissue specific effects
Endpoints	Assesses a full complement of toxic and inflammatory responses	May fail to detect subtle effects or may find effects (e.g., haemorrhage or neovascularisation) not included in scoring
Statistics	Enables summary table and graphs	Precision of individual scores is not often stated
External validity	Gives good predictive accuracy with few false negatives; provides protective margin by overestimating human reactivity	Interspecies differences can limit generalizability
Data uses	Can be incorporated into regulatory requirements	Databases listing different compounds are not necessarily comparable

**1.4 Organisations researching *in vitro* alternatives to the Draize test**

Many organisations are striving to develop and validate alternatives to animal tests. The European Centre for the Validation of Alternative Methods (ECVAM) was established by the European Commission (EC) with the aim of consolidating European efforts towards the development and validation of alternatives. The Interagency Co-ordinating Committee on the Validation of Alternative Methods (ICCVAM) co-ordinates activities on the validation of alternatives in the United States, and the Fund for the Replacement of Animals in Medical Experiments (FRAME) implements the Three R's, of Russell and Burch (1959), research strategy and encourage realistic consideration of ethical and scientific issues involved in the use of laboratory animals.



## 1.5 Reduction, refinement and replacement (The Three R's)

There is an increasing realisation of the limitations of animal models in relating and predicting human adverse reaction and/or toxicology, as fundamental differences between species are identified (Wilson, 2000; Putnman *et al.*, 2002). Social and scientific criticism of *in vivo* experimentation is beginning to exert pressure on governments to introduce tighter legislation to limit the number of animals used in scientific research. One approach that is widely accepted, is the Three R's (Russell and Burch, 1959), in their book 'Principles of Humane Experimental Technique' as reduction, refinement and replacement.

- **Reduction** : the most immediate prospects for improving the current situation are to reduce the number of animals used e.g. improved experimental design and the use of *in vitro* pilot studies to assess the need for animal experimentation (Russell and Burch 1959).
- **Refinement** : amending procedures already in use so that long-term animal suffering is minimised (Russell and Burch 1959).
- **Replacement** : replacing of animal experiments as and when suitable alternatives are developed. Validated according to certain criteria outlined in ECVAM reports (Balls *et al.*, 1995; Spielmann *et al.*, 1997; Spielmann *et al.*, 1998; Spielmann *et al.*, 2000) is now an integral part of acceptance within the scientific community and by the regulating bodies involved.

## 1.6 Current *in vitro* alternatives to the Draize eye irritation test

Before the Draize test can be totally replaced in acute local ocular irritancy testing, *in vitro* assays need to be validated. This involves the establishment of a common protocol and a prediction model (required to convert *in vitro* results into measurement of irritancy related to the Draize results or known human effects), and to assess their interlaboratory reproducibility. For full validation a multi-laboratory trial where coded substances are assessed by the *in vitro* methods and compared to reliable *in vivo* data to evaluate their suitability for safety assessments is required (Balls and Karcher 1995). The alternative methods to the Draize eye irritation test comprise organotypic models such as isolated eyes or components thereof (Taliana *et al.*, 2001), tissue (Harbell *et al.*, 1991) and cell culture systems (Chiba *et al.*, 1999) and physicochemical tests (Naughton *et al.*, 1989). Success in developing and validating alternative tests to replace the Draize rabbit eye irritation test has remained elusive despite major efforts by the European Centre for the Validation of Alternatives (ECVAM), industry trade associations, individual companies and academia. Five major validation studies took place between 1991 and 1997. These were the EC/HO study (Balls *et al.*, 1995), CTFA study (Gettings *et al.*, 1996), COLIPA study (Brantom *et al.*, 1997), IRAG study (Bradlaw *et al.*, 1997) and the MHW/JCIA study (Ohno *et al.*, 1999). These studies focused on a small number of alternative methods including; the hen's egg-chorioallantoic membrane method (HET-CAM) (Leupke, 1985), chorioallantoic membrane-trypan blue staining method (CAM-TB) (Hagino *et al.*, 1999), red blood cell haemolysis method (RBC) (Okamoto *et al.*, 1990), haemoglobin denaturation method (HD) (Hayashi *et al.*, 1993), Skin2™ (ZK1100 model) (Harbell *et al.*, 1991), MATREX™ (Gay *et al.*,

1992), CornePack (Torishima *et al.*, 1990), SIRC cells using crystal violet as an endpoint of cytotoxicity (SIRC-CVS) (Itagaki *et al.*, 1991), SIRC cells using neutral red uptake (SIRC-NRU) (Okamoto *et al.*, 1990), HeLa cells using MTT reduction (HeLa-MTT) (Mosmann, 1983), CHL cells using crystal violet staining (CHL-CVS) (Ishidate and Odashima, 1977; Gordon and Bergman, 1987), the MDCK cells using fluorescein leakage (FLA; Tchao, 1988; Shaw *et al.*, 1990), using a specific set of test chemicals, usually cosmetic ingredients as employed by the MHW/JCIA study (table 1.05). Draize tests were conducted according to the OECD guidelines using the same test substances as were evaluated in the alternative *in vitro* assays tests (Ohno *et al.*, 1999).

**Table 1.05 : Range of test substances with corresponding Draize data used in the multilaboratory validation study (adapted from Ohno *et al.*, 1999).**

Substance No.	Compound Name	Class	Maximal Average Score (MAS)
1	Isotonic sodium chloride solution		0
2	Polyoxyethylene hydrogenated castor oil	Surfactant (nonionic)	0
3	Tween 20	Surfactant (nonionic)	0.7
4	Polyethyleneglycol monolaurate	Surfactant (nonionic)	3.3
5	Sodium N-lauroyl sacosinaye (30% solution)	Surfactant (anionic)	10.3
6	Sodium hydrogenated tallow-L-glutamate	Surfactant (anionic)	26.7
7	Sodium dodecyl sulphate	Surfactant (anionic)	16
8	Sodium polyoxyethylene laurylether sulphate (27% solution)	Surfactant (anionic)	10
9	Polyoxyethylene octylphenylether	Surfactant (nonionic)	41.3
10	Benzalkonium chloride	Surfactant (cationic)	78
11	Sucrose fatty acid ester	Surfactant (nonionic)	11
12	Glycerin	Polyols	0
13	Acid Red 92	Colour additives	25
14	Polyoxyethylene sorbitan monooleate	Surfactant (nonionic)	0
15	Calcium thioglycolate	Organic salts	4
16	Distearyldimethylammonium chloride	Surfactant (cationic)	ND*
17	2-Ethylhexyl-p-dimethylamino benzoate	PABA derivatives	0
18	Cetylpyridinium chloride	Surfactant (cationic)	ND
19	Methyl p-hydroxybenzoate	Esters	ND
20	Isopropyl myristate	Esters	0.7
21	Polyethylene glycol 400	Polyols	0
22	Silicic anhydride	Inorganics	ND
23	Benzyl alcohol	Alcohols	23
24	Sodium salicylate	Organic salts	0
25	m-Phenylenediamine	Amines	4.3
26	Ethanol	Alcohols	0
27	Monoethanolamine	Alkanolamines	23.3
28	Triethanolamine	Alkanolamines	0
29	Stearyltrimethylammonium chloride	Surfactant (cationic)	91.3
30	Diisopropanolamine	Amines	23
31	Potassium laurate	Surfactant (anionic)	38
32	Cethyltrimethylammonium bromide	Surfactant (cationic)	76.7
33	Acetic acid	Carboxylic acids	68
34	Butanol	Alcohols	34
35	Chlorhexidine gluconate solution (20% solution)	Organic salts	28.3
36	Dorniphen bromide	Surfactant (cationic)	96.3
37	Lactic acid	Carboxylic acids	9.7
38	Glycolic acid	Carboxylic acids	25
39	Di(2-ethylhexyl) sodium sulfosuccinate	Surfactant (anionic)	57

### **1.6.1 Chorioallantoic membrane (CAM) test**

The Hens egg test-chorioallantoic membrane (HET-CAM) method first described by Leupke (1985), has been extensively studied and is considered validated in various research facilities (Hagino *et al.*, 1999). This method is designed to examine the microscopic changes in the chorioallantoic membrane (CAM), such as hyperaemia, haemorrhage and coagulation, following treatment with test substances (Leupke 1985; Hagino *et al.*, 1999). It has been demonstrated (Hagino *et al.*, 1999) that the HET-CAM assay shows a useful correlation between *in vitro* results and the Draize irritation assay. However, Reinhardt *et al.*, (1987) reported that the HET-CAM test was not fully predictive for eye irritation. Hagino *et al.*, (1999) concluded that the HET-CAM tests may be useful for predicting and evaluating eye irritation despite problems of inter-laboratory reproducibility.

### **1.6.2 Red blood cell (RBC) haemolysis test**

The standard haemolysis test evaluates membrane injury using erythrocytes as a model biological membrane and measures the amount of haemoglobin that leaks from the erythrocyte. The Okamoto *et al.*, (1999) concluded that the haemolysis method was useful as an alternative to the Draize eye irritation assay for the prediction and evaluation of severe (MMAS  $\geq 50$ ) irritation based on damage to the cell membrane (Okamoto *et al.*, 1999).

### 1.6.3 Skin2™ ZK1100 and tissue equivalent assay

Skin2™ ZK1100 (ZK1100) and Tissue Equivalent (TEA) assays are human dermal models constructed with cultured human cells to model the tissue structure relevant to the human eye or dermal tissue (Harbell *et al.*, 1991) evaluated as alternatives to the Draize eye irritation assay. Naughton *et al.*, (1989) reported that human dermal fibroblasts were grown in a three dimensional nylon mesh matrix. This model is referred to as Skin2™ ZK1100 cultures. Triglia *et al.*, (1991) reported the application of another artificial skin model for detecting skin irritation. In contrast to the previous model, this model contains co-cultured stratified squamous epithelium and stromal fibroblasts, derived from skin and grown on nylon mesh. The stratified squamous epithelium is air-interfaced to promote epithelial layering but is non-cornified. Hence, the cells in the model are cultured in a manner that stimulates mucosal epithelium, such as the cornea. This model was referred to as the tissue equivalents model during the validation study that concluded that both the ZK1100 and TEA may be developed as valid alternatives to the Draize test (Kurishita *et al.*, 1999). Kurishita *et al.*, (1999) concluded that both assays have the capacity to predict the eye irritation of test substances, however, further analysis would be required to define the utility of the ZK1100 and TEA assays. When employed with cosmetic ingredients (Brantom *et al.*, 1997) in the COLIPA/EU validation study such models were considered highly predictive.

#### **1.6.4 MATREX™**

MATREX™ is a test system for evaluating eye irritation potential, using the living dermal model (LDM). The LDM consists of normal human fibroblast in a contracted collagen lattice, which eventually forms a three-dimensional structure (Ohuchi *et al.*, 1999). Ohuchi *et al.*, (1999) concluded that the MATREX™ assay could be useful as part of a battery of tests in predicting the eye irritation potential of exogenous compounds.

#### **1.6.5 CornePack**

The cytotoxicity test of neutral red uptake (NRU) in normal rabbit corneal epithelial cells (CornePack) was evaluated as a potential alternative method to the Draize rabbit eye irritation test. Uchiyama *et al.*, (1999) demonstrated several characteristic limitations of the CornePack assay. Although CornePack appears to be useful for the preliminary screening for cytotoxicity of substances due to its high sensitivity and ease of operation, Uchiyama *et al.*, (1999) concluded this technique demonstrated difficulties in evaluating substances such as anionic detergents, acids, alkanolamines or alcohols.

#### **1.6.6 Rabbit cornea (SIRC) cell cytotoxicity**

Two common assays, the neutral red uptake assay (SIRC-NRU) and the crystal violet staining assay (SIRC-CVS), were evaluated by Tani *et al.*, (1999) as an alternative to the Draize eye irritation assay. Tani *et al.*, (1999) concluded: the intra- and

interlaboratory reproducibility of the SIRC-NRU/CVS method was relatively good for most test substances including dyes and water-insoluble substances; the results of the study demonstrated a good correlation with *in vivo* results; accurate evaluation of strong acids, alkanolamines and alcohols is difficult; SIRC-NRU and SIRC-CVS show similar efficacy in predicting irritancy potential.

#### **1.6.7 Cervical epitheloid carcinoma (HeLa) cell cytotoxicity**

The cytotoxicity test using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay on HeLa cells (MTT-HeLa) demonstrated several limitations, particularly for acids, amines and alcohol. However, overall the results of MTT-HeLa showed good reproducibility and strong correlation with *in vivo* data (Chiba *et al.*, 1999).

#### **1.6.8 Chinese hamster lung (CHL) cell cytotoxicity**

The CHL cell line cell toxicity test employs crystal violet staining as a measurement of cell toxicity. Among the cells used in this validation study, SIRC cells were the only established cell line derived from rabbit corneal cells (Ohno *et al.*, 1999). Tani *et al.*, (1999) indicated that the SIRC cell method did not show any significant differences from methods employing other cell types e.g. HeLa (Chiba *et al.*, 1999) or CHL (Okumura *et al.*, 1999). The validation study concluded that cytotoxicity tests utilising CHL gave almost the same results as those using the other cell types (Ohno *et al.*, 1999). Okumura *et al.*, (1999) reported that although the assay could



accurately predict the toxicity of exogenous irritants, definitive criteria were required to establish the compounds that could be applied to the model.

### **1.6.9 Fluorescein leakage assay**

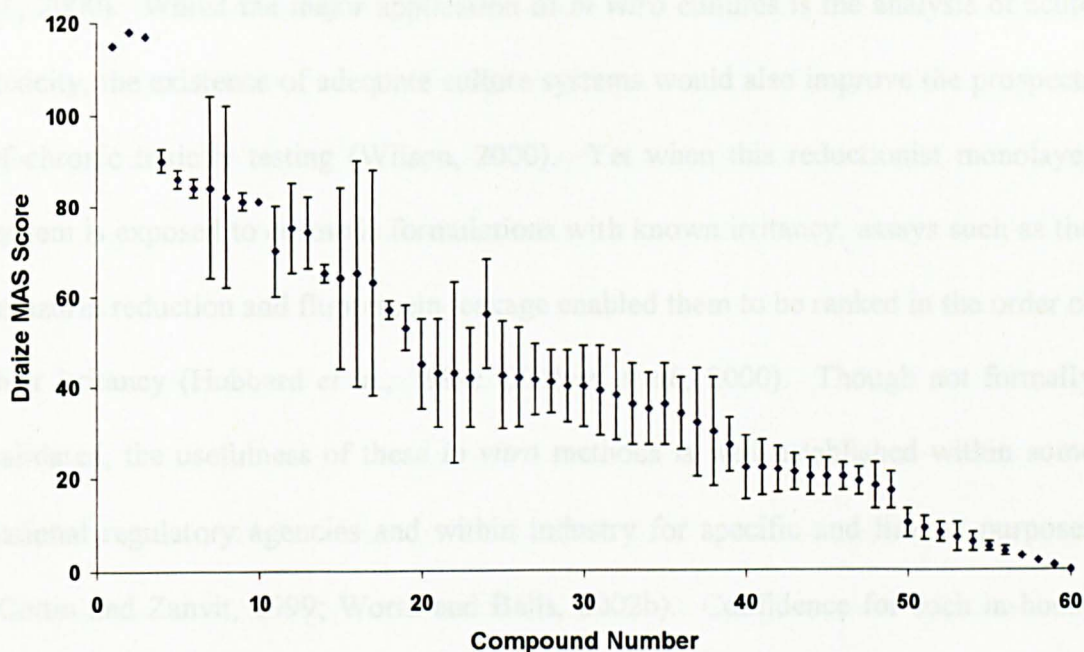
The method employed was based upon that of Tchao (1988). The loss of trans-epithelial impermeability of a confluent layer of MDCK cells was measured following a 1 minute exposure to the test chemical. One of the greatest difficulties was the removal of viscous, non-toxic chemicals due to the problems associated with their removal following the exposure period (Balls *et al.*, 1995). In addition Balls *et al.*, (1995) reported a number of limitations with the fluorescein leakage assay, including; only materials which are in solution or suspension may be tested, incompatibilities between test chemicals and the insert material resulting in leakage due to insert damage rather effects on the MDCK monolayer (Balls *et al.*, 1995) and chemical interaction with the insert membrane prolonging exposure times (Ward *et al.*, 1997a). Although the COLIPA/EU validation study evaluated the performance of the fluorescein leakage assay and demonstrated good interlaboratory reproducibility between the laboratories and a reasonable statistical fit within its prediction model, there was insufficient data to assess its reliability (Brantom *et al.*, 1997).

### **1.7 Limitations of *in vitro* alternatives to the Draize test**

Generally the accuracy of an *in vitro* model is assessed by identifying a number of materials that have previously been tested for irritation *in vivo* and then retesting the

same materials in an *in vitro* assay (Eun and Suh, 2000). Data is conventionally analyzed by plotting the *in vitro* data on one axis and the animal data on the other. The mathematical relationship between the *in vitro* and *in vivo* scores, i.e., the algorithm that allows one to predict an *in vivo* score from an *in vitro* score, is then used to generate the prediction model, i.e. how the *in vitro* results from a new material could be used to derive a Draize equivalent score. The Draize eye irritation test, does demonstrates a high degree of variation for individual test compounds across multiple laboratories (figure 1.01). This affects the ability of an *in vitro* assay to correlate with *in vivo* data. None of the assays (HET-CAM, CAM-TB, RBC, HD, Skin2<sup>TM</sup> (ZK1100 model), MATREX<sup>TM</sup>, CornePack, SIRC-CVS, HeLa-MTT or CHL-CVS) of the MHW/JCIA study were found capable of replacing the Draize test, but some of the assays demonstrated considerable promise as screens for ocular irritancy. In the COLIPA/EU study the conclusion was that the 3D skin model and the fluorescein leakage were worthy of further investigation and refinement, whilst there were reservations about the other tests included (Brantom *et al.*, 1997). The main reason for this is the difficulty of comparing *in vitro* test results with historical animal data where the subjective scoring of tissue lesions in the eye in the Draize test provides variable estimates of eye irritancy.

**Figure 1.01** : Representation of the mean $\pm$ S.D of the most reliable Draize eye irritation MMAS scores of the 60 chemicals used in the EC/HO validation trial (adapted from Balls *et al.*, 1995).



Other possible contributing reasons for the failure of recently completed validation studies are: a) the *in vitro* tests only partially modelled the complex *in vivo* eye irritation response, b) the protocols were insufficiently developed, and c) the choice of statistical approaches for analysing the data was not appropriate (Balls *et al.*, 1999). It was clear that the use of a cell line to model basal cell toxicity could accurately predict the more potent eye irritancy verging on corrosion (MMAS 80 – 120), in that this cell death is the endpoint regardless of compounds or chemicals. Several techniques to replace the Draize eye test in the mild to moderate band (MMAS 0 – 30) relying on the use of cells that express tight junctions and control of paracellular penetration pathways (e.g. the Madin-Derby canine kidney (MDCK) or corneal epithelial cells) and including specific non-lethal endpoint assays (e.g.

fluorescein leakage alone or including the resazurin reduction or neutral red uptake assays) to evaluate both the functionality and activity/viability of these cells subsequent to chemical exposure (Hubbard *et al.*, 1994; Clothier and Samson, 1996; Grant and Acosta, 1996; Kruszewski *et al.*, 1997; Clothier *et al.*, 1999; Clothier *et al.*, 2000). Whilst the major application of *in vitro* cultures is the analysis of acute toxicity, the existence of adequate culture systems would also improve the prospects of chronic toxicity testing (Wilson, 2000). Yet when this reductionist monolayer system is exposed to cosmetic formulations with known irritancy, assays such as the resazurin reduction and fluorescein leakage enabled them to be ranked in the order of their irritancy (Hubbard *et al.*, 1994; Clothier *et al.*, 2000). Though not formally validated, the usefulness of these *in vitro* methods is well established within some national regulatory agencies and within industry for specific and limited purposes (Cottin and Zanvit, 1999; Worth and Balls, 2002b). Confidence for such in-house use of some of the currently available *in vitro* methods is dependent on the availability of appropriate benchmark chemicals, historical information on similar materials, an understanding of the limitations of the assay(s) and the technical expertise of the user. In addition this historical information for cosmetic companies is based on human experience of the action of formulations or the relating of human skin irritancy with chemicals to predict levels that can be incorporated into cosmetic formulations that may encroach the eye. For these reasons, such use of *in vitro* methods is often company-specific, e.g. L'Oreal and Boots Plc have now incorporated the fluorescein leakage assay into their pre-clinical safety assessments (Cottin and Zanvit 1997; Zanvit *et al.*, 1999). The rationale for pursuing the development of a human ocular based *in vitro* alternatives is to provide greater confidence in the prediction of human reactions to mild and moderate chemicals and

formulations, in particular with respect to babies and those with “sensitivities” to certain ingredients. In addition to provide a species specific model it is desirable that these assays afford the potential to express mechanistically relevant responses that can be used for predicting human ocular toxicity and recovery potential. These objectives address the recently stated needs for mechanism-based (Balls and Clothier, 1992; Frazier, 1994; Technical Committee on Alternatives to Animal Testing, ILSI, 1996) and human cell-based tests (Kruszewski *et al.*, 1995) for supporting *in vitro* toxicology and human risk assessment.

## **1.8 Chronic repeat exposure in vitro**

The development of alternatives to the use of animals for toxicity testing have mainly focused upon developing *in vitro* tests for quantitative measurement of acute local toxicity (Fentem and Balls, 1992) following a single high-dose exposure (EC/HO (Balls *et al.*, 1995), CTFA (Gettings *et al.*, 1996), COLIPA/EU (Brantom *et al.*, 1997), IRAG (Bradlaw *et al.*, 1997), MHW/JCIA (Ohno *et al.*, 1999)). A substantial proportion of the studies have been carried out using undifferentiated secondary cell lines grown in a monolayer in flask culture. In these cases, a range of general and some specific indicators of toxic effects can be measured (Hanley *et al.*, 1999). While cell culture cannot adequately mimic whole body responses to toxin challenge, there are a number of advantages with such approaches including ease of use, cost, the numbers of experiments which are feasible, the range of concentrations that can be tested and complexity of the test substances (Soto *et al.*, 1998). The degree of toxicity of any exposure is a function of; the dose which target cells receive, the duration of the exposure and the ability of the exposed cells to recover from the

exposure (Combes, 1999). However, little account is taken of the potential role of long-term effects in modulating the toxic response (Combes, 1999). Thus, it would seem logical to develop *in vitro* chronic exposure assays that will allow the monitoring of changing responses during the exposure period, and which also permit the measurement of recovery levels. Repeat dosing to obtain chronic exposures can lead to increased resistance to toxicity due to induction of repair processes and detoxification enzymes, as well as saturation of intracellular targets (Soto *et al.*, 1998; Combes, 1999). On the other hand, decreased resistance could result as a consequence of the induction of activating thereby increasing the concentration of reactive chemical species at target sites (Soto *et al.*, 1998; Combes, 1999). Numerous chemicals exert toxic effects, at concentrations that in an acute assay would be non-cytotoxic, following chronic low-dose treatments. There are several different ways in which humans are exposed chronically or repeatedly e.g. dietary intake of foodstuffs and washing formulations, as well as splashings in the eye, involve exposing populations of cells, which could be in different stages of recovery from earlier responses (Combes, 1999).

## **1.9 Development of chronic exposure models**

The limited published data on *in vitro* chronic toxicity models is mainly due to technical problems which must be overcome to develop such systems, including; effects on cell maintenance (e.g. trypsinisation, detachment from supporting matrix, reattachment, stabilization and regrowth (Hanley *et al.*, 1999)), attachment matrix, culture senescence and non-lethal cytotoxicity assays (Combes, 1999). Several advances have been made in protocols for studying cellular recovery from toxic

injury, and also for long-term culturing and repeat dosing. These advances include; the availability of improved methods for culturing and exposing cells, such as synthetic porous membranes (Combes, 1999) and the development of both non-lethal viability and function assays e.g. resazurin reduction and fluorescein leakage assays respectively (Clothier and Samson, 1996; Clothier *et al.*, 1999). Other important advances include better methods for retention of cell differentiation status and stability in culture to enhance longevity, delaying culture senescence using continuous and immortalized cell lines, by applying different culture conditions (MacDonald, 1993; Tatenos and Yoshizato, 1996; Barile, 1997). ECVAM reviewed the use of long term toxicity methods in a report (Balls *et al.*, 1999). Many of the problems associated with chronic toxicity modelling of epithelial barriers *in vitro* were addressed by the study of Clothier and Samson (1996) using a continuous cell line that expressed tight junctions grown on a synthetic porous membrane. It is also important that epithelial cells, with an inherent polarity express this *in vitro*. The advantage of the porous membrane is that they facilitate topical or systemic application of a variety of test materials for toxicity studies, since the epithelial cells have distinct basal and apical compartments. The use of membrane inserts effectively provides a means whereby the culture system can be divided into an upper and lower compartment that can mimic topical or systems exposure. Employing assays using non-cytotoxic endpoints, effects at times before and after initiation of treatment can be monitored (e.g. combined fluorescein leakage-resazurin reduction assay), and repeat exposure of the cells to fresh test material can be undertaken (Clothier and Samson 1996; Combes, 1999). Using such a model Clothier and Samson (1996) observed that repeat treatment of monolayer cultures of MDCK cells, with surfactants lead to cumulative, dose dependent effects on barrier

function that was related to effects on zona-occulden adhesion molecules lost and restitution. However, the study of Clothier and Samson (1996) was restricted to measuring the effects on MDCK viability and barrier function of an acute 1 minute exposure with subsequent measurements made over 96 hours to ascertain recovery. *In vitro* chronic exposure assays need to be designed with the ability to monitor changing responses during the exposure period. Techniques should be adaptable, since the mechanisms of toxicity could change with the dose, cell target and number of exposures (Clothier *et al.*, 1997). Assays could adopt different treatment regimens, for example ranging from daily re-exposure for a fixed time, as is adopted *in vivo* with human patch testing of cosmetics, to repeat exposure once recovery has occurred from the previous insult as described for the evaluation of repeat exposure to mild surfactants (Clothier and Samson, 1996). It is also important to relate what happens *in vivo* in terms of the initial and recovery effects to those *in vitro* mechanisms. Thus knowledge of the structure of the human eye is important as it does differ from the rabbit eye.

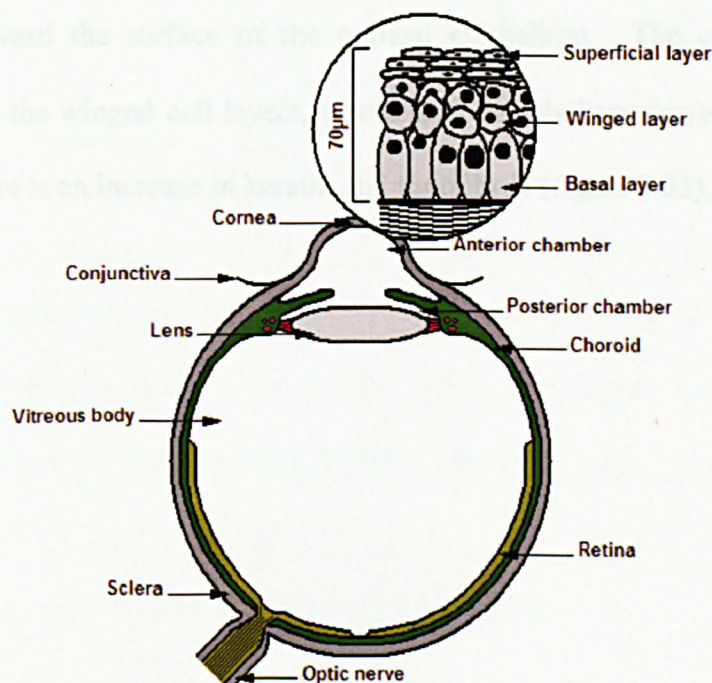
#### **1.10 Anatomical structure of the human eye**

The eye is a highly specialised organ of photoreception, a process which involves the conversion of different quanta of light energy into nerve action potentials. The adult human eyeball measures approximately 2.5cm in diameter and of its total surface area, only the anterior one-sixth is exposed, the remainder is recessed and is protected by the orbit into which it fits. Anatomically, the wall of the eyeball can be divided into three distinct layers: vascular tunic, retina tunic and fibrous tunic (Totoro and Grabowski 1996) (figure 1.02). The fibrous tunic is the superficial coat



of the eyeball. It consists of the anterior cornea and posterior sclera. The cornea is an avascular, transparent coat that is separated from the coloured iris by the anterior chamber. Its outer surface consists of nonkeratinised stratified squamous epithelium. The middle coat of the cornea consists of collagen fibres and fibroblasts, and the inner surface is simple squamous epithelium (Totora and Grabowski, 1996).

**Figure 1.02 :** Representation of the right eye demonstrating the relationship between the sclera, choroid and retina with enlargement demonstrating the layers of the corneal epithelium.

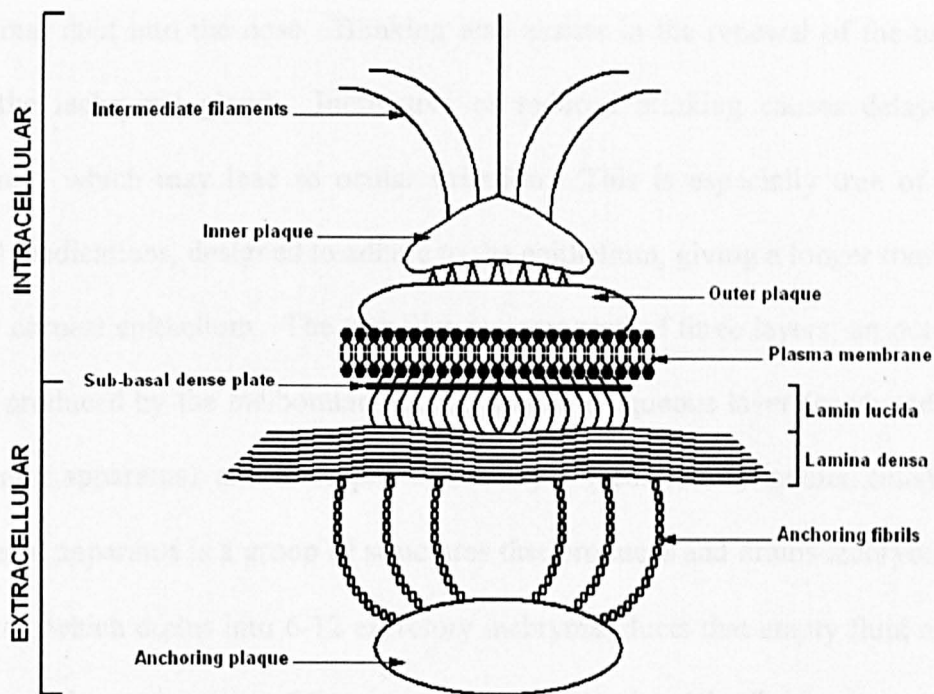


### 1.10.1 The cornea

The cornea is comprised of five layers: the multilayered epithelium, Bowmans layer, the thick stroma consisting of keratinocytes and orthogonally arranged collagen lamellae, Descemets membrane, and the endothelium. The corneal epithelium has 5-

8 layers of cells continuous with the bulbar (ocular) conjunctiva and is 70 $\mu$ m thick (figure 1.02). The basal layer secretes an irregular basal lamina to which it attaches by hemidesmosomes. Basal layer integrity is essential for re-epithelialisation after corneal damage (Klyce and Beuerman, 1988). The periphery of the basal layer is attached to a thicker basal lamina by basal villi, and is more mitotically active than the rest of the epithelium (Fone and Yanoff, 1972). Epithelial cells in the cornea are continuously in motion (Ladage *et al.*, 2002) in two principle directions: centripetal and vertical. It has been demonstrated (Auran *et al.*, 1995; Ladage *et al.*, 2002) under normal conditions basal cells migrate centripetally at a speed of 1.7 to 32 $\mu$ m a day. In contrast, vertical movement occurs when basal cells leave the basal lamina and move toward the surface of the corneal epithelium. The cells then move anteriorly into the winged cell layers, where their metabolism decreases. The cells flatten and there is an increase in keratin and tonofibrils (figure 1.03).

**Figure 1.03 :** Schematic representation of the adhesion complex of human corneal epithelium (adapted from Zagon *et al.*, 2001).



The corneal epithelium provides the eye with a smooth wettable anterior surface covered by the tear film (Sugrue and Zieske, 1997). This in part is achieved through the actions of the lachrymal apparatus producing the tear film.

### 1.10.2 The tear film and the lachrymal apparatus

When a foreign substance enters the eye from the external environment, it will first reach the pre-corneal tear film. This thin film covers the conjunctiva and cornea and has many vital functions. It corrects faults in the cornea to provide a smooth refractive surface and acts as a lubricant to reduce friction during eye movements and blinking. It also holds some oxygen and nutritional molecules for the cornea, has an antibacterial action and helps flush out surface debris. Immediately after blinking,

the tear film is around 9µm thick, this reduces to a minimum of 4µm after around 30 seconds, after which the film will break up unless a blink occurs. Blinking normally occurs about 15-20 times a minute in humans, with tears draining away, via the lachrymal duct into the nose. Blinking also assists in the renewal of the tear film from the lachrymal gland. Ineffective or reduced blinking causes delayed tear clearance, which may lead to ocular irritation. This is especially true of certain topical medications, designed to adhere to the epithelium, giving a longer transit time on the corneal epithelium. The tear film is composed of three layers; an outer lipid layer (produced by the meibomian gland), a middle aqueous layer (produced by the lachrymal apparatus) and a deeper mucin layer (secreted by goblet cells). The lachrymal apparatus is a group of structures that produces and drains lachrymal fluid (pH 7.4), which drains into 6-12 excretory lachrymal ducts that empty fluid onto the surface of the conjunctiva of the upper eyelid. From here the fluid passes medially over the anterior surface of the eyeball to enter the lachrymal puncta. Tears then pass into two ducts, the lachrymal canals, which lead into the nasolachrymal sac and then into the nasolachrymal duct. Lachrymal fluid is a watery solution containing salts, some mucus, and a bactericidal enzyme called lysozyme. The fluid cleans, lubricates, and moistens the eyeball. Normally tears are cleared away as fast as they are produced by evaporation or by passing into the lachrymal canals and subsequently into the navel cavity. If an irritating substance contacts the conjunctiva, however, the lachrymal glands are stimulated to oversecrete and fluid can accumulate, leading the eyes to “water”. This is a protective mechanism, since tears dilute and wash away the irritating substance. The lachrymal gland secretion rate is partly controlled by its parasympathetic and sensory innervation. The space behind the cornea and surrounding the lens is filled with a clear fluid called the

aqueous humor which supplies the lens and cornea with nutrients. This fluid is secreted by the processes of the ciliary body which is a component of the vascular tunic. Although the aqueous humor is isotonic with the blood, it is not a simple ultrafiltrate of plasma as it is relatively rich in bicarbonate. Moreover, inhibitors of the enzyme carbonic anhydrase decrease its rate of production. The aqueous humor drains through a fibrous mesh situated at the junction between the cornea and the sclera into the canal of Schlemm and from there into the venous blood. The pressure within the eyeball is about 2kPa (15mmHg). This is known as the intraocular pressure and it serves to maintain the rigidity of the eye, which is essential for keeping the focal plane and distance constant for sharp image formation. This pressure is sustained by keeping the balance between production and drainage of aqueous humor constant. If the drainage is obstructed, the pressure rises and a condition known as glaucoma results. In glaucoma, the retinal circulation can become occluded, leading to retinal damage and defective vision. In glaucoma, the excessive production of aqueous humor cannot be vented through the front of the eye due to the histological structure of the cornea. The cornea acts as a barrier to the outflow of water from the interior of the eye and the passage of pathogens into the internal structures of the eye, via tightly adherence of the cells to one another as well as the underlying matrix.

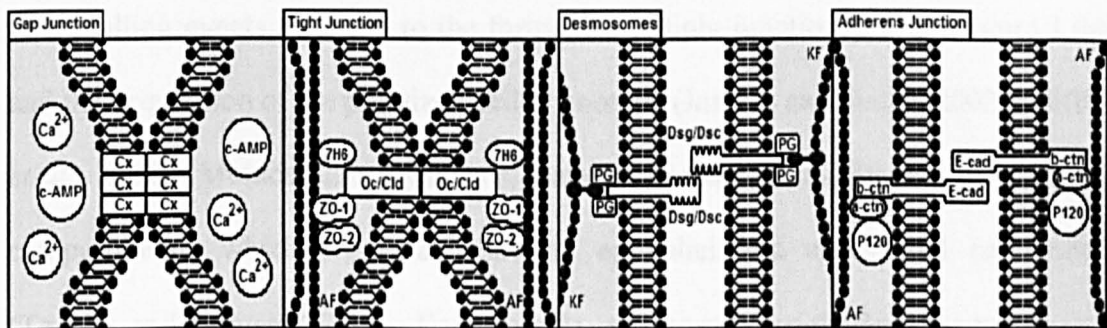
### **1.10.3 Barrier function of the human cornea**

In order to accomplish their barrier functions, epithelial must demonstrate a structural and functional polarity characterised by a distinctive organisation of the



cytoskeleton and by an asymmetric distribution of the membrane protein between the apical and basolateral domains (Chifflet *et al.*, 2004).

**Figure 1.04 :** Schematic illustration of intercellular junctions found in various tissue of the body (adapted from Suzuki *et al.*, 2003).



c-AMP, cyclic adenosine monophosphate; Cld, claudin; Oc, occludin; ZO-1 and ZO-2, zonula occludens-1 and -2; 7H6, 7H6 antigen; AF, actin filament; Dsg, desmoglein; Dsc, desmocollin; DP, desmoplakin; PG, plakoglobin; KF, keratin filament.

During morphogenesis, mechanical forces generated by the dynamic rearrangements of cell-cell contacts and the cytoskeleton modulate changes in cell shape and motility (Jamora and Fuch, 2002). Cell contraction is directly associated with phosphorylation of myosin light chains (MLC) by the calcium/calmodulin (CaM)-dependent myosin light chain kinases (MLCK) (Garcia *et al.*, 1995). MLCK activation which modify the fibrous actin (f-actin) cytoskeleton have been shown to be strongly linked to permeability increase (Haselton 1992; Yu and Gotlieb, 1992; Garcia *et al.*, 1995). Transmembrane members of the cadherin superfamily of intercellular adhesion proteins have a pivotal function in these processes. E-cadherin forms cell-cell contacts through homotypic interactions, which results in the formation of stable junctions. The extracellular domains of E-Cadherin dimerise and

cluster in a calcium-dependent manner, triggering an association of the cytoplasmic tails of cadherin to the actin cytoskeleton network (Jamora and Fuch, 2002). The recruitment of  $\alpha$ - and  $\beta$ -catenin is required for this cytoskeleton linkage, which in turn is essential for the stabilization and formation of E-cadherin-mediated cell-cell junctions referred to as adherens junctions (AJ's) (Yap *et al.*, 1997; Jamora and Fuch, 2002). This interaction with the actin cytoskeleton initiates a complex cascade of signalling events that lead to the formation of tight junctions (TJ's) (figure 1.04) and the acquisition of the polarized cell phenotype (Jamora and Fuchs, 2002; Chifflet *et al.*, 2004). Multicellular organisms contain various compositionally distinct fluid compartments, which are established by epithelial and endothelial cell sheets (Tsukita and Furuse, 1999). For example, the corneal epithelium functions as a barrier that isolates the eye from the outside environment (Ban *et al.*, 2003). For these cell sheets to function as barriers maintaining the distinct internal environment of each compartment the paracellular pathway between adjacent cells in the sheet must be tightly sealed or controlled to prevent the unwanted diffusion of solute and solvents, whilst the apical and basolateral domains must be differentiated to allow active transport across the confluent sheet of selected materials (Tsukita and Furuse, 1999). Tight junctions are directly involved in paracellular sealing (barrier function) providing a continuous seal around the apical aspect of adjoining epithelial cells, thereby, preventing the free passage of molecules between adjacent epithelial cells (paracellular pathway). Recent studies reveal that the TJ complex includes two integral transmembrane proteins: claudin; and occludin and membrane associated proteins such as zonula occludins-1 (ZO-1), ZO-2 and ZO-3 (Tsukita *et al.*, 2001).

### 1.10.3.1 Zonula Occludins 1 (ZO-1)

ZO-1 with a molecular mass of 220kDa (Stevenson *et al.*, 1986; Lapierre 2000) was one of the first proteins identified and localized to the TJ where in conjunction with ZO-2, -3 (Lapierre 2000) acts as a scaffolding or plaque protein responsible for connecting the proteins occludin and the claudins to the f-actin cytoskeleton (figure 1.04) (Citi, 1993; Anderson, 2001). The f-actin cytoskeleton is a highly dynamic network, changing structure during the cell cycle and in response to extra- and intracellular signals (Hall, 1994). It has been demonstrated (Sugrue and Zieske, 1997) that the expression and localization to the interior surface of the plasma membrane of ZO-1 is largely influenced by calcium dependent interactions with the cytoskeleton. There is accumulating evidence that some unique proteins constitute TJ's. Collectively these membrane associated proteins are thought to interconnect the tight junction to the submembraneous cytoskeleton network (Stevenson *et al.*, 1986; Anderson *et al.*, 1993; Citi, 1993; Tsukita and Furuse, 1999). It now appears that ZO-1 is a ubiquitous component of the tight junction in all epithelia and endothelia (Sugrue and Zieske, 1996) interacting with occludin (Stevenson *et al.*, 1986; Citi, 1993; Tsukita and Furuse, 1999) an integral membrane protein involved in the permeability barrier function of the tight junction. However, it has been shown that ZO-1 may be found at non-tight junctional locations (Schnabel *et al.*, 1990; Howarth *et al.*, 1992; Miragall *et al.*, 1994) leading to the unfortunate misinterpretation that the presence of ZO-1 immunoreactivity equates to the presence of tight junctions (Sugrue and Zieske, 1996).



### 1.10.3.2 Occludin

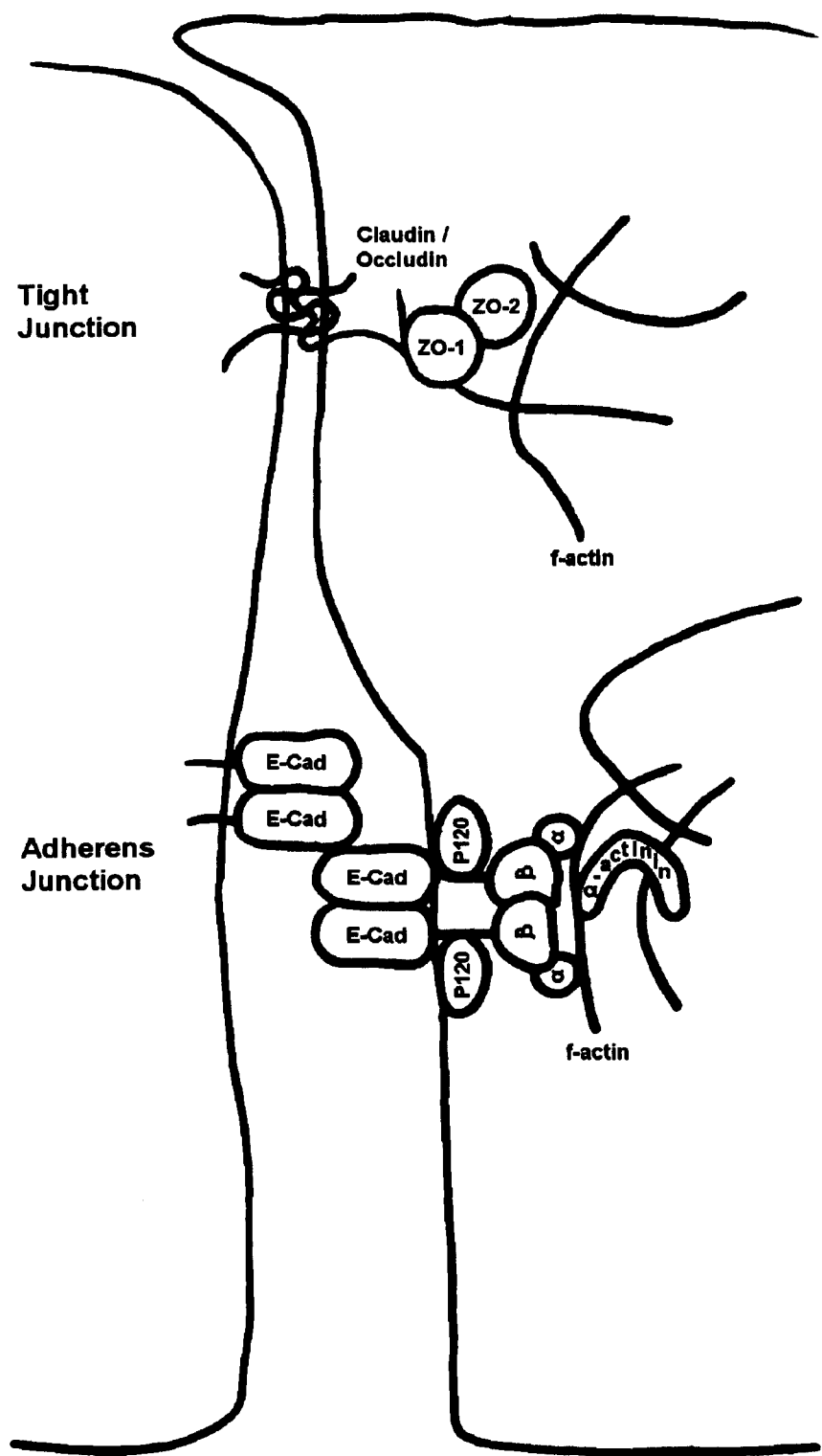
Occludin was the first transmembrane protein of tight junctions to be identified (Furuse *et al.*, 1993; Balda and Matter 2000). Occludin is a 60 – 65kDa protein with four transmembrane domains (Furuse *et al.*, 1993; Balda and Matter 2000; Ban *et al.*, 2003) with both N- and C- termini oriented into the cytoplasm (Balda and Matter 2000). Occludin appears to be the most important and relatively well characterized transmembrane protein of the tight junction (Kale *et al.*, 2003) with the C-terminal interacting *in vitro* with several intracellular scaffolding proteins including ZO-1, ZO-2, ZO-3 (Furuse *et al.*, 1993; Balda and Matter 2000; Ban *et al.*, 2003) and f-actin (Goodenough, 1999; Wittchen *et al.*, 1999). To date there have been no reports of occludin isoforms – that is occludin-related genes – although two forms of occludin are generated by alternate splicing (Morita *et al.*, 1999; Tsukita and Furuse, 1999). The level of occludin expression in various cell types correlates well with the number of tight junction strands (Saitou *et al.*, 1997; Tsukita and Furuse, 1999).

### 1.10.3.3 Claudin-1

Claudins, 23kDa integral membrane proteins bearing four transmembrane domains, have been identified as components of TJ strands (Furuse *et al.*, 1998; Tsukita and Furuse, 2002), and to be directly involved in intercellular sealing in simple epithelia (Balda and Matter, 2000; Anderson 2001; Tsukita *et al.*, 2001; Furuse *et al.*, 2002; Ban *et al.*, 2003). Claudin-1 and -2 were originally identified because they cofractionated with occludin from sonicated fractions (Furuse *et al.*, 1998). There is no evidence for a distinct interaction between occludin and claudin (Balda and

Matter, 2000), and claudins do not copurify with occludin from detergent solubilised membranes (Balda and Matter, 2000). At least 24 members of the claudin family have been identified (Furuse *et al.*, 1998; Gow *et al.*, 1999; Tsukita and Furuse, 2002). In contrast to occludin, individual claudins are generally expressed in only a restricted number of specific cell types, suggesting that they are associated with tissue-specific functions of tight junctions (Balda and Matter, 2000) but the level of claudin expression does not correlate with the number of tight junction strands (Saitou *et al.*, 1997; Tsukita and Furuse, 1999). Besides their membrane topology, different claudin members share other structural features, suggested by the observation that eight different claudins can bind to the three submembrane proteins ZO-1, ZO-2 and ZO-3 by interaction involving the claudins C-terminal cytosolic domain and the PDZ domain of the submembrane plaque proteins (Simon, 1999).

**Figure 1.05 :** Interactions between molecular components of the tight and adherens junctions.



#### **1.10.4 Innervation of the cornea in repair mechanism**

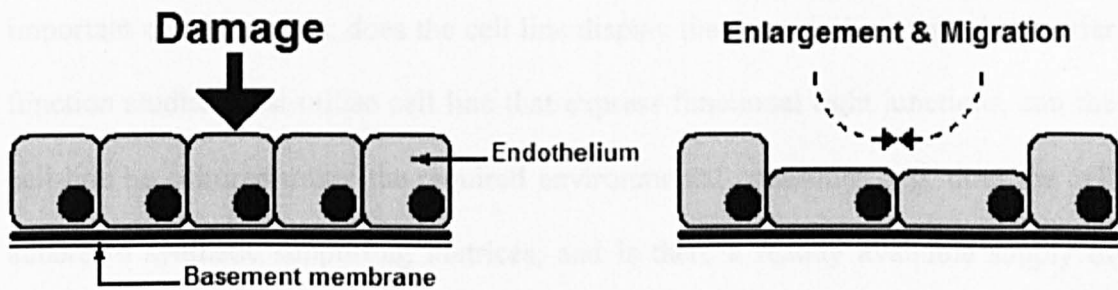
The cornea is one of the most densely innervated tissues in the body and is richly supplied with sensory and autonomic nerve fibres (Muller *et al.*, 2003). The subject of corneal innervation has taken heightened importance in recent years due to the observation that corneal nerves are routinely injured following modern refractive surgical diseases or following certain corneal diseases (Muller *et al.*, 2003). This damage can lead to transient or chronic neurotrophic deficits. In addition, in the past several years neuropeptides synthesised by corneal nerve fibres have been used successfully to promote corneal wound healing of corneas resistant to conventional therapy (Muller *et al.*, 2003). Despite the numerous studies published in recent years that have indicated that cytokines, growth factors, and neuropeptides can influence epithelial proliferation and differentiation *in vitro* (Lambiase *et al.*, 2000), a precise therapeutic approach to modulate the healing process has not yet been identified.

#### **1.10.5 The corneal endothelium and wound healing**

The corneal endothelium, a monolayer of cells at the posterior side of the cornea, is crucial for maintaining the transparency of the cornea. It consists of a single layer of cells forming a boundary between the corneal stroma and the anterior chamber (Joyce, 2003). It regulates the ion composition of the whole cornea, permitting optimal hydration of the cornea, and thus constant thickness and transparency. Consequently a disturbance of endothelial functions can provoke cornea oedema followed by partial or complete loss of transparency (Hoppenreijs *et al.*, 1996). Apart from loss of endothelial cells during aging, other factors such as contact lens

wear, ultraviolet radiation, accidental trauma (physical and chemical), inflammation or dystrophy (Doughty, 1989; Nucci *et al.*, 1990; Williams *et al.*, 1992) can lead to a decrease in endothelial cell density and to an abnormal cell mosaic which can compromise the functional integrity of the endothelial monolayer (Hoppenreijds *et al.*, 1996). Many cellular events occur during corneal wound healing to ensure that the tissue can resume its normal function of light refraction (Taliana *et al.*, 2001). When both the epithelium and the underlying stroma are wounded, epithelial cells and corneal fibroblasts (CF's) are directly involved in the repair process (Taliana *et al.*, 2002; Joyce 2003). In the wounded stromal tissue CF's are activated and transform the into myofibroblast (MF) phenotype (Taliana *et al.*, 2001) to effect wound closure by contraction of the stroma under repair. In severe injury, if the endothelial cell layer is penetrated or damaged the clinical outcome may be in doubt. Both cell proliferation and migration contribute to the formation of the endothelium from neural crest-derived mesenchymal cells (Joyce, 2003). There is evidence to indicate that once the mature endothelial monolayer has formed, proliferation ceases and cells remain non-replicative throughout the normal lifespan (Taliana *et al.*, 2001; Joyce, 2003) and recovery from damage is achieved through cell enlargement and migration rather than division (figure 1.06).

**Figure 1.06 :** Endothelial cell enlargement and migration as a response to severe injury to the primate corneal endothelium.



During the first stage of wound healing, drastic changes in morphometric parameters of corneal endothelial cells take place: pleomorphism (variation in cell shape) and polymegethism (variation in cell size). After initial coverage of the wound areas, a period of cell transformation and rearrangement occurs (Hoppenreijns *et al.*, 1996). Cell transformation continues to take place for many years after recovery from a wound area damaged during surgery such as keratoplasty or lens extraction (Mishima, 1982). In other species such as rodents and rabbits, the wounded corneal endothelium is regenerated by cell replication in addition to enlargement and migration (Hoppenreijns *et al.*, 1996). Due to the tremendous regenerative capacity in these species, the endothelial layer is restored almost to its normal state (Landshman *et al.*, 1988; Ling *et al.*, 1988). However, the low mitotic capacity of the primate corneal endothelium precludes complete regeneration of the endothelial layer after surgical intervention or trauma (Haung *et al.*, 1989; Hoppenreijns *et al.*, 1994).

### 1.11 Selection of a specific cell type for an *in vitro* model

The selection of a cell line for *in vitro* toxicity testing depends upon a number of important considerations; does the cell line display the correct phenotype, i.e. barrier function studies must utilise cell line that express functional tight junctions, can the cell line be cultured under the required environmental conditions, e.g. does the cell adhere to synthetic supporting matrices, and is there a readily available supply of cells for high throughput screens, e.g. continuous cell line or a reliable source of primary human cells. To replace the Draize eye test an *in vitro* system must include a model of the barrier function of the corneal epithelium. A widely utilized rabbit corneal cell line, SIRC (Statens Seruminstitut rabbit cornea cells), was characterized ultrastructurally and immunohistologically (Niederhorn *et al.*, 1990). SIRC cells lack desmosomes, cytoplasmic filaments, and cytokeratin-structures that are characteristic of human corneal epithelial cells. By contrast, the dendritic morphology, presence of vimentin, and the extensive dense accumulations of ribosomes and rough endoplasmic reticulum are consistent with a fibroblastic phenotype. Although SIRC cells are often described as being of epithelial origin collectively, the morphology, ultrastructural features, and antigenic composition favour the hypothesis that SIRC cells are fibroblastic cells (keratocytes) and not corneal epithelial cells (Niederhorn *et al.*, 1990). In contrast Madin-Darby canine kidney (MDCK) cells grown to confluency as a monolayer have been used *in vitro* to model epithelial barrier function (e.g. Tchao, 1988; Shaw *et al.*, 1990; Hubbard *et al.*, 1994; Zanvit *et al.*, 1999) in conjunction with the combined fluorescein leakage-resazurin reduction assay (Clothier and Samson, 1996).

One problem with the *in vitro* alternatives to the Draize test is that many do not reflect the multilayer nature of the corneal epithelium in both humans and animals (Clothier *et al.*, 2000). The extent to which this precise nature of the barrier in the human corneal epithelial layer is required *in vitro* is not yet fully defined, but a true physiological model should be multilayered as is the *in vivo* cornea. Two continuous corneal cells lines, the Japanese human corneal epithelium (J-HCET) cells and Gillette human corneal epithelial cells (HCE-T) can grow and form a four- to six-layer epithelium permeable supports given the correct stimulus (Kahn *et al.*, 1993; Kruszewski *et al.*, 1995; Ward *et al.*, 1997b; Clothier *et al.*, 2000) allowing for the development of both a monolayer and three dimensional (3D) model of the human cornea *in vitro*. The HCE-T or J-HCET cell line are considered as the preferred basis of an *in vitro* corneal model. However, it is essential to ensure that the phenotype of a transformed corneal cell line remains unaltered, and whilst the HCE-T cell line is stable to passage 20, the J-HCET is considerably more stable up to 100 passages (Araki-Sasaki *et al.*, 1995) and has been genetically typed by LGC for the FRAME laboratory.

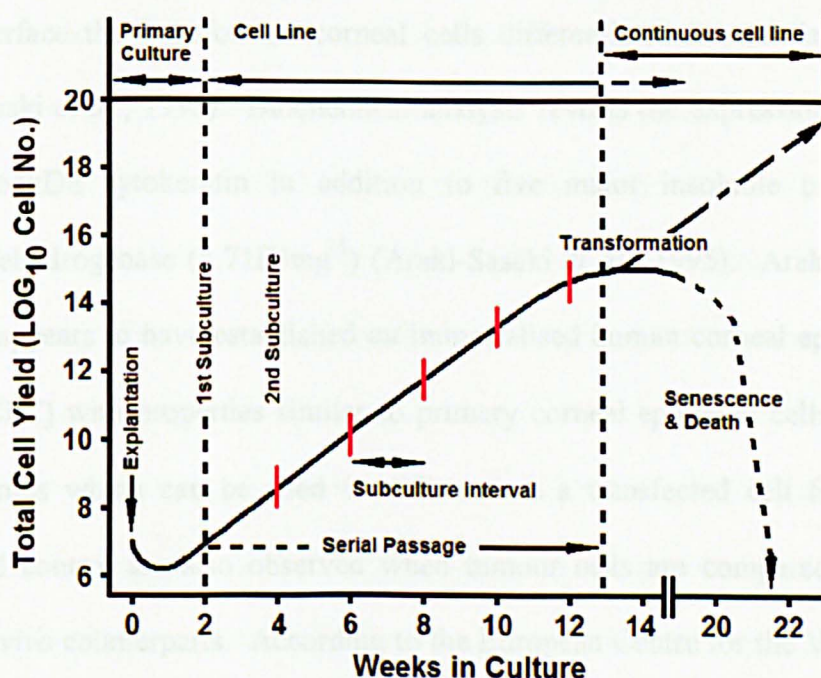
#### **1.11.1 Transfected human corneal epithelial cells**

Many protocols exist for the isolation and limited *in vitro* propagation of primary mammalian cells, including those of the corneal origin (Baum *et al.*, 1979; Kahn *et al.*, 1993; Engelmann and Friedl 1989; Araki-Sasaki *et al.*, 1995; Bednarz *et al.*, 1998). Primary cultures of human corneal epithelial cells, especially from adult donors undergo upto 20 cumulative population doublings before entering senescence (figure 1.07). Furthermore, the characteristic morphology is often lost during



prolonged culturing (Albert *et al.*, 1972; Turksen *et al.*, 1989; Engelmann and Bohnke, 1990) when a cell line becomes transformed. The limited volume of material and the variability between cell cultures from different donor eyes makes it very difficult to perform routine biochemical and physiological investigations on primary human corneal epithelial cells (Bednarz *et al.*, 2000). To overcome this problem primary cell cultures may be transfected *in vitro* to increase cell proliferation and longevity.

**Figure 1.07 :** Evolution of a cell line. The vertical axis represents total cell growth for a hypothetical cell culture. Total cell number is represented on the y-axis on a log scale and time in culture, on the x-axis on a linear scale. Although a continuous cell line is depicted as arising at 13 weeks it could arise at any time. Likewise senescence could arise at any time (adapted from Freshney 1994).



The most common method for immortalisation of human cells whilst retaining a specific phenotype is transfection with the Simian virus 40 (SV40) genome which encodes the large T-antigen (Bednarz *et al.*, 2000; Ozdarendeli *et al.*, 2003). Simian virus (SV40) originated in Asian rhesus monkeys and is closely related to the human polyomaviruses. SV40 can induce tumours in rodents and can transform human cells *in vitro*. The transforming ability of SV40 is dependent on the expression of the early region of the viral genome, coding for the T antigen (Tag), a nuclear phosphoprotein with multiple biological and biochemical properties (Sladek and Jacobberger, 1992; Ozdarendeli *et al.*, 2003). The effect of SV40 transfection is to decrease the duration of G1 whilst increasing G2 and M phases with a net increase in cell proliferation (figure 1.06). Araki-Sasaki *et al.*, (1995) isolated human corneal epithelial cells which were subsequently transfected with SV40 to obtain a continuous growing cell line (J-HCET). This cell line was reported to grow for more than 400 generations exhibiting a cobblestone-like appearance similar to normal corneal epithelia in culture (Araki-Sasaki *et al.*, 1995). When cultured at the air-liquid interface the transformed corneal cells differentiated in multilayer fashion (Araki-Sasaki *et al.*, 1995). Biochemical analysis reveals the expression of cornea-specific, 64kDa cytokeratin in addition to five major insoluble proteins and aldehydedehydrogenase ( $8.71\text{IUmg}^{-1}$ ) (Araki-Sasaki *et al.*, 1995). Araki-Sasaki (*et al.*, 1995) appears to have established an immortalised human corneal epithelial cell line (J-HCET) with properties similar to primary corneal epithelial cells. Many of the properties which can be used to differentiate a transfected cell from a non-transfected control are also observed when tumour cells are compared with their normal *in vivo* counterparts. According to the European Centre for the Validation of Alternative Methods (ECVAM) review of *in vitro* methods (Worth and Balls,

2002b), models need to match human *in vivo* expression of differentiated function with the relevant highly differentiated cell phenotypes. Therefore, in order to assess the potential use the J-HCET cell line has in modelling *in vitro* chronic corneal toxicity it is necessary to establish the growth rate, phenotypic stability during prolonged culture and presence and correct localisation of proteins associated with the adherens and tight junctions, particularly since different media have been used in their culture with or without serum (Araki-Sasaki *et al.*, 1995; Combes *et al.*, 1999; Araki-Sasaki *et al.*, 2000; Moore *et al.*, 2005).

## **1.12 Growth media to culture human corneal epithelial cells**

The validity of the cultured cell as a model of physiological function *in vivo* has frequently been criticised (Freshney, 1994). There are problems of characterisation due to the altered cellular environment; cells proliferate *in vitro* that would not normally *in vivo*, cell-cell and cell matrix interactions are reduced because purified cell lines lack the heterogenicity and the three dimensional architecture found *in vivo*, and the hormonal and nutritional milieu is altered. The provision of the appropriate environment, nutrients, hormones and substrate is fundamental to the expression of specialised function (Engelmann and Friedl, 1989; Freshney, 1994). *In vitro* cell, tissue and organ cultures play an important role in science, medicine and industry (Van der Valk *et al.*, 2004). The growth of many cell types in culture requires serum in the medium (Castro-Munozledo and Hernandez-Quintero, 1997) as a source of hormones, cytokines, vitamins, attachment, spreading and growth factors (Tezel and Del Priore, 1998) the absence of which can have profound effects upon *in vitro* cultures (Tezel and Del Priore, 1998). There are disadvantages to the use of animal

derived materials such as serum, including the potential contamination with infectious agents such as mycoplasma, viruses (e.g. Hepatitis, HIV) and prions (Scrapie, Bovine Spongiform Encephalopathy) which may prove detrimental to the viability of cell cultures and pose a health risk to individuals exposed to the contaminated medium or supplements. In addition animal derived materials are ill defined supplements, and thus are ambiguous factors in cell culture (Barnes *et al.*, 1987) since it has been demonstrated that batches display quantitative and qualitative variations in their composition (Price and Gregory 1982). Since *in vitro* methods have been, and are being developed as alternatives to animal experiments, the use of bovine serum can seem to be contradictory in some ways to the purposes of the Three R's concept (Russell and Burch, 1959). With the identification, cloning and recombinant production of essential growth factors several very effective chemically defined, serum free media have been formulated. Thus the development of serum free cell culture medium has been stimulated (Taub, 1990; Bjare, 1992; Ranaldi *et al.*, 2003; Fitzsimmons *et al.*, 2003; Van der Valk *et al.*, 2004) and increasing numbers of formulations for the culture of primary isolates and continuous cell lines are being developed (Taub, 1990; Bjare, 1992; Fitzsimmons *et al.*, 2003) although serum free media are generally more cell type specific (Tezel and Del Priore, 1998).

### **1.13 *In vitro* cell viability and function assays**

The rationale behind using *in vitro* cytotoxicity assays to predict *in vivo* toxicity stems from the concept of 'basal cell cytotoxicity' proposed by Ekwall (1983). It was suggested that for most chemicals, toxicity is a consequence of non-specific alterations in cellular functions (Ekwall, 1983; Evans *et al.*, 2001). Evaluating the

cytotoxic potential of compounds may, therefore, give an indication of their toxic potential *in vivo* (Evans *et al.*, 2001). To this end a number of international studies have been performed previously to assess the correlation between cytotoxicity and *in vivo* toxicity in a wide range of cell lines with varying degrees of success (Knox *et al.*, 1986; Clothier *et al.*, 1988; Fry *et al.*, 1990; Garle *et al.*, 1994). *In vitro* cytotoxicity assays must fulfil a number of criteria, they must be simple, rapid, efficient, reliable, sensitive, reproducible, safe, and allow a cost effective measurement of cell viability. Importantly, the *in vitro* assay selected must not interfere with the compound to be tested (O'Brien *et al.*, 2000) and have a clearly defined end-point. To validate (i.e. to establish the scientific validity of (Balls *et al.*, 1995)) an alternative test, it is necessary to demonstrate that for its stated purpose: the test system has a sound scientific basis; the predictions made by the prediction model are sufficiently accurate; and the results generated by the test system are sufficiently reproducible within and between laboratories, and over time. These conditions can be referred to as the criteria for scientific relevance, predictive relevance, and reliability, respectively (Worth and Balls, 2002a).

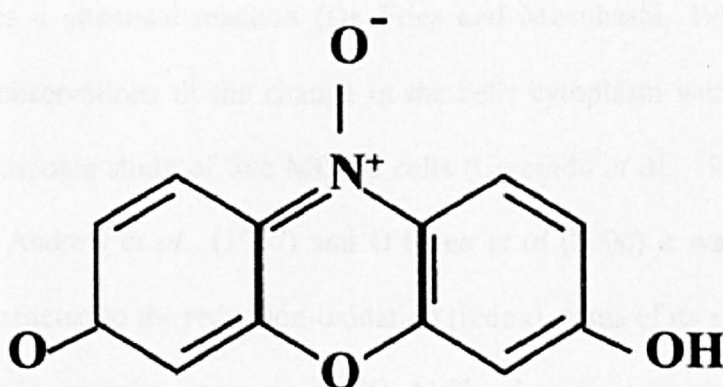
### **1.13.1 Cell viability assays**

#### **1.13.1.1 The Resazurin reduction (Alamar Blue™) assay**

The Alamar Blue™ (Resazurin Reduction) assay (Page *et al.*, 1993) has been compared to other viability assays and demonstrated a good correlation in predicting cytotoxicity, e.g. with the trypan blue exclusion-(Gazzano-Santoro *et al.*, 1997), and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide salt (MTT)- assay

(Black *et al.*, 1999) whilst allowing for repeated or continuous measurements (Goegan *et al.*, 1994; Vian *et al.*, 1994; Clothier and Samson, 1996; O'Brien *et al.*, 2000) which cannot be achieved with some assays such as the MTT assay due to its inhibition of metabolic processes and final solubilisation step. Recent studies (Rasmussen, 1999; O'Brien *et al.*, 2000) comparing the Alamar Blue™ and resazurin (7-Hydroxy-3H-phenoxazin-3-one-10-oxide) assays concluded that Alamar Blue™ is essentially resazurin dye (figure 1.08) supplemented with stabilizing agents.

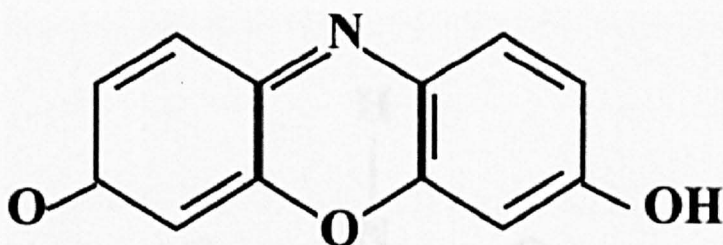
**Figure 1.08** : Non-reduced Alamar Blue™ corresponds to resazurin.



Resazurin is a tetrazolium-based dye incorporating resazurin and resorufin as oxidation-reduction indicators that yield colorimetric changes and a fluorescent signal (when excited at a specific wavelength of 530nm) in response to metabolic activity. In an irreversible reaction, the blue non-fluorescent oxidised form (resazurin) becomes pink and fluorescent (resorufin, figure 1.09) upon reduction (O'Brien *et al.*, 2000; Byth *et al.*, 2001).

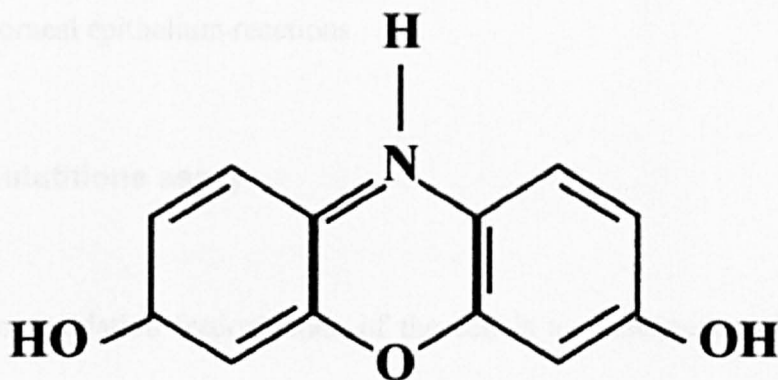


**Figure 1.09** : Reduced Alamar Blue™ corresponds to resorufin.



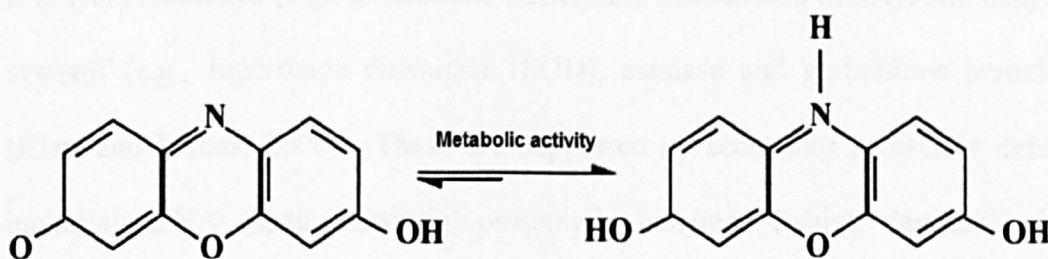
It has been arbitrarily postulated (De Fries and Mistuhashi, 1995) that resazurin is reduced by mitochondrial activity, although uncertainty as to whether resazurin reduction actually occurs intracellularly, at the plasma membrane surface or just in the medium as a chemical reaction (De Fries and Mistuhashi, 1995). This was settled when observations of the change in the cells cytoplasm were reported in a confocal microscopic study of live MDCK cells (Cereijido *et al.*, 1980). In studies conducted by Andrew *et al.*, (1997) and O'Brien *et al* (2000) it was demonstrated that resazurin reacted to the reduction-oxidation (redox) status of its environment. In the absence of living cells, resazurin in CO<sub>2</sub> buffered medium was not converted to resorufin, however, in the presence of primary rat hepatocytes fluorescence, and therefore resorufin was clearly produced (Andrews *et al.*, 1997). Further intracellular reduction (two-electron reduction) of resorufin yields a colourless product dihydroresorufin (figure 1.10), which, if the fluorescence based assay was employed, would be suggestive of a lower resazurin reduction i.e. measurements and subsequent interpretation of fluorescence would imply that the cells were less active.

**Figure 1.10** : Further intracellular reduction of resorufin yields a colourless product, dihydroresorufin.



This phenomenon has been known for a long time as being due to the extensive reduction of resazurin (blue) into resorufin (pink) and then to dihydroresorufin (colourless) (figure 1.11) (Erb and Ehlers 1950; O'Brien *et al.*, 2000).

**Figure 1.11** : Further metabolic activity is responsible for the reversible reduction of resorufin to dihydroresorufin.



The lower the concentration of resazurin within the assay solution the more rapidly it is completely reduced and becomes colourless i.e. resazurin concentration is a limiting factor. Hence, being aware of this possible problem and the possible effects of proteins on the resazurin reduction (Page *et al.*, 1993) the assay is normally



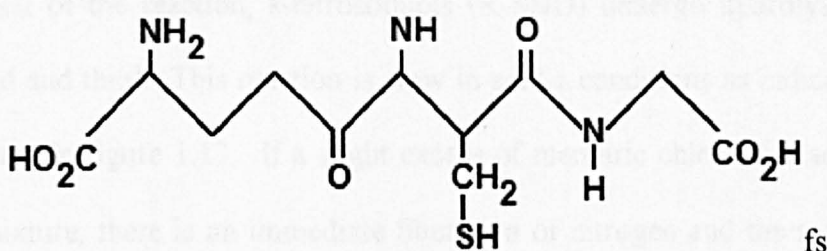
conducted in excess resazurin. The non-cytotoxic nature of this assay makes it an attractive candidate for the repeat assessment of cell activity. However, other detoxification pathways need to be included to encompass the possible mechanisms of adverse corneal epithelium reactions

#### **1.13.1.2 Glutathione assay**

The reduction-oxidation (redox) state of the cell is a consequence of the balance between the levels of oxidising reactive oxygen species (ROS) and reducing equivalents. Elevation of ROS in excess of the buffering capacity and enzymatic activities designed to modulate ROS levels result in potentially cytotoxic “oxidative stress” (Pastore et al., 2003) a situation in which the cellular redox homeostasis is altered because of excessive production of ROS and/or impairment of cellular antioxidant mechanisms. To prevent oxidative damage and allow survival in an oxygen environment mammalian cells have developed two important defence mechanisms: a thiol reducing buffer consisting of proteins with redox active sulphhydryl moieties (e.g., glutathione (GSH) and thioredoxin (TRX)) and enzymatic systems (e.g., superoxide dismutase (SOD), catalase and glutathione peroxidase) (Klatt and Lamas, 2000). These are supported by secondary protective defences, including DNA repair enzymes, proteolytic enzymes (which degrade oxidised proteins) and the radical scavenging vitamins E and C (Klatt and Lamas, 2000). The antioxidant mechanisms present within mammalian cells provide protection from a specific range of ROS e.g. catalase and glutathione peroxidase convert hydrogen peroxide into harmless products, SOD acts upon superoxide anions and glutathione-S-transferase detoxifies DNA hydroperoxides (Chandra *et al.*, 2000). Glutathione

(figure 1.12) (L-γ-glutamyl-Lcysteinylglycine) is the principle non-protein thiol (-SH) involved in the antioxidant cellular defence (Pastore *et al.*, 2003). It is a tripeptide composed of glutamic acid bound to cysteine through its γ-CO<sub>2</sub>H group and glycine.

**Figure 1.12 :** Reduced Glutathione is a tripeptide consisting of glutamic acid, cysteine and glycine (Adapted from Pastore *et al.*, 2003).

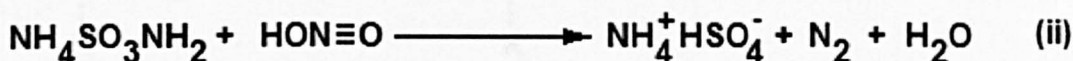
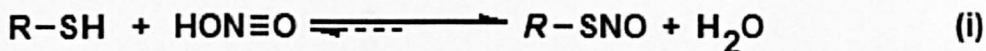


Glutathione is a ubiquitous molecule that is produced in all organs, especially the liver. Glutathione exists in two forms, the antioxidant “reduced glutathione” (GSH) and oxidized glutathione known as glutathione disulphide (GSSG).

#### 1.13.1.2.1 Chemistry of GSH<sub>Eq</sub> assay

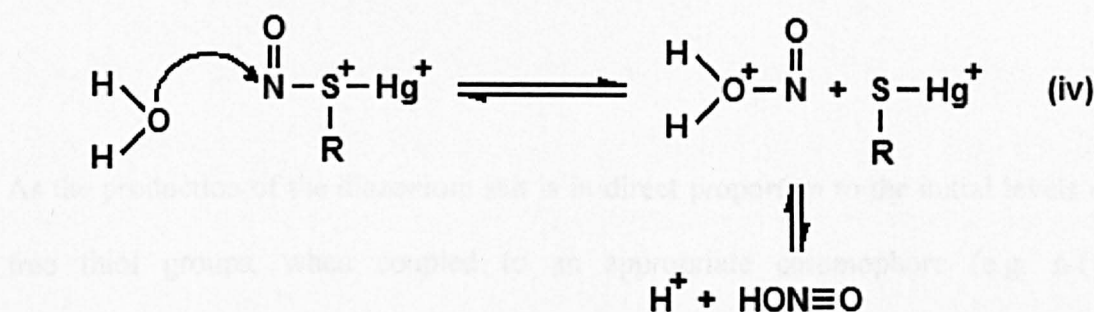
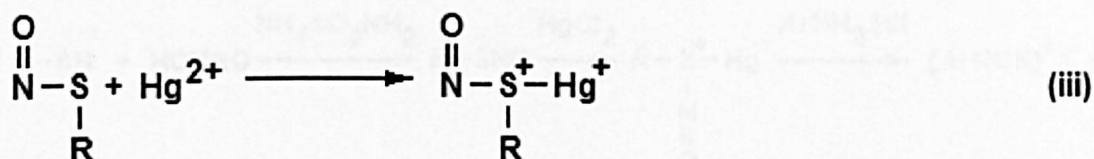
In the presence of nitrous acid, the free thiol group (R-SH) found on the amino acid cysteine is converted to its s-nitrosothiol derivative (R-SNO) (figure 1.13).

**Figure 1.13** : Conversion of thiol groups to their s-nitroso derivatives (i) in the presence of nitrous acid. Ammonium sulphamate is used to remove excess nitrous acid (ii) adapted from (Saville, 1958).



In a reversal of the reaction, s-nitrosothiols (R-SNO) undergo hydrolysis to form nitrous acid and thiol. This reaction is slow in acidic conditions as indicated by the broken arrow in figure 1.13. If a slight excess of mercuric chloride is added to the reaction mixture, there is an immediate liberation of nitrogen and the red colour of the s-nitrosothiol compound is rapidly destroyed. This is due to the coordination of the metal cation with the nitrosothiol (figure 1.14) weakening the N-S bond (iii) increasing its susceptibility to nucleophilic attack by water molecules (iv)

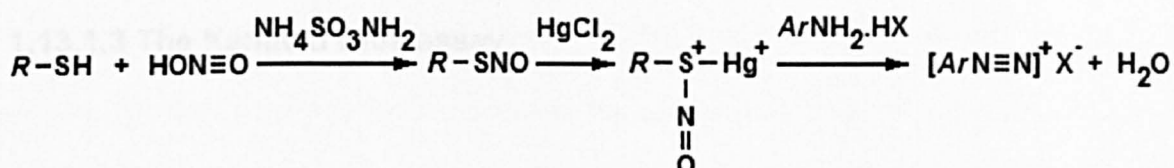
**Figure 1.14** : An excess of mercuric chloride results in an immediate liberation of nitrogen and the destruction of the red colour of the compound adapted from (Saville, 1958).



During these reactions the liberated nitrous acid reacts with the ammonium sulphamate to form gaseous nitrogen. If sulphanilamide is mixed with the mercuric salt before addition to the s-nitrosothiol solution, the amine competes favourably with the sulphamate for the nitrous acid equivalent and gives a high yield of the corresponding diazonium salt (figure 1.15).

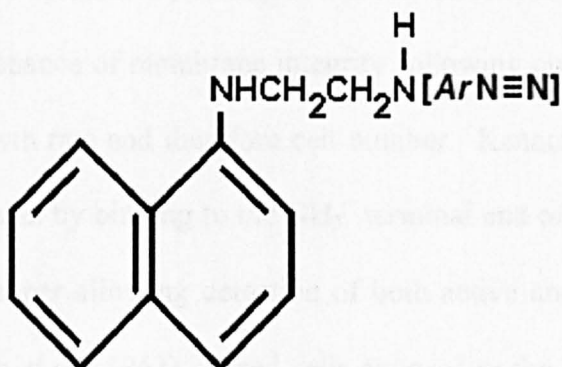


**Figure 1.15 :** Sulphanilamide mixed with the mercuric salt before addition to the s-nitrosothiol solution gives a high yield of the corresponding diazonium salt.



As the production of the diazonium salt is in direct proportion to the initial levels of free thiol groups, when coupled to an appropriate chromophore (e.g. n-(1-naphthyl)ethylenediamine) it can be used as a colorimetric scheme for the determination of thiols within a specimen (figure 1.16).

**Figure 1.16 :** The diazonium salt is coupled to n-(1-naphthyl)ethylenediamine to yield an intensely coloured dye.



Whilst changes in redox potential as measured via the resazurin and GSH assays are important, they need to be related to cell number and a total cell protein assay was employed.

#### **1.13.1.3 The Kenacid Blue assay**

With the ever increasing ability to accurately measure minute quantities of specific molecules of interest through the use of enzyme linked immunosorbant assay (ELISA), radiological immunoassay (RIA) and various molecular biology tools, a limiting factor can be a common denominator for comparison of samples (Starcher, 2001). Total protein has been one of the most universal denominators and several methods have been devised to measure protein in the microgram quantities (Lowry *et al.*, 1951; Bradford, 1976). The kenacid blue total protein assay utilises a dye (Coomassie blue or Kenacid blue) that reversibly binds to cellular protein components, which is proportional to the total number of cells present within a culture (Knox *et al.*, 1986; Balls and Clothier, 1992; Clothier *et al.*, 1992). The basis of the assay for use in cytotoxicity testing is that any effects, for example, on protein production or maintenance of membrane integrity following chemical treatment will result in altered growth rate and therefore cell number. Kenacid blue or Coomassie blue dye detects protein by binding to the  $\text{NH}_3^+$  terminal end of the protein molecule in a non-specific manner allowing detection of both active and non-active proteins (Fazekas de St Groth *et al.*, 1963). Fixed cells exposed to the dye will bind the dye in proportion to the amount of protein present within the culture. This relates to the total cell number (Knox *et al.*, 1986) and the total protein content of the culture may then be calculated from the standard curve (Gray *et al.*, 1999). Cell distribution

within a well can be viewed macroscopically prior to desorbing the dye giving an instant indication of the assays success. If necessary measurements can also be repeated on the same cultures, by retaining the cultures, following dye desorption. The chemical nature of a substance may place constraints on this technique. Other problems which may arise include false indications of the amount of cell protein present if culture media is not aspirated sufficiently; resulting in the formation of a ring of dried protein around the well at the air/liquid interface. It is also possible for the dye to precipitate out, producing an uneven distribution, constant gently shaking and less handling time minimises this. It is also important to consider that the toxicity of a chemical may be underestimated by this technique as apoptotic cells remain attached to the well can be detected. If run too soon after exposure to the test substance the necrotic cells may not have been detached and so would be included with the live cells. Also the chemical may bind to the dye as well, but a no cell blank control treated with the top concentration of chemical can be used to check this. Hence the total protein assay has been used in conjunction with a cell viability assay e.g. neutral red uptake assay (Riddell *et al.*, 1986).

#### **1.13.1.4 The neutral red uptake assay**

The neutral red uptake (NRU) assay (Borenfreund and Puerner, 1985) is a cytotoxicity assay based upon the inability of dead and damaged cells to actively take up the dye neutral red. Neutral red is selectively retained in the cytoplasm, in particular the lysosomes of living cells, by differential pH of the lysosome and surrounding cytoplasm. The amount of neutral red taken up is directly proportional to the number of viable cells in the culture as long as the chemical under

investigation does not directly affect the pH of the lysosomes e.g. as with the antimalaria drug Chloroquine (Riddell *et al.*, 1986).

### **1.13.2 Cell function assays**

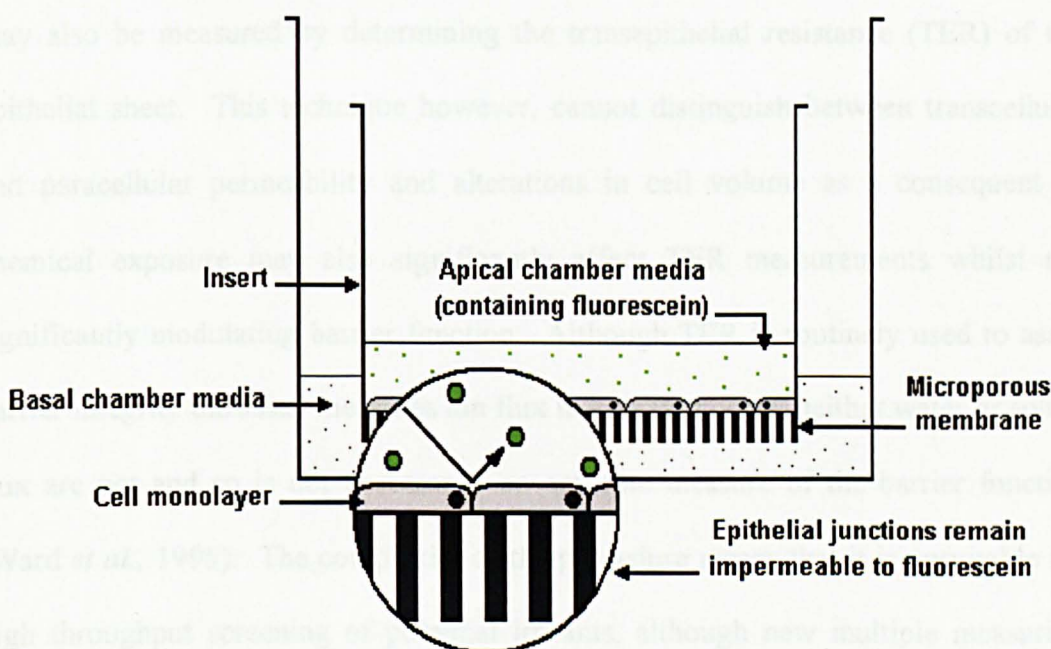
#### **1.13.2.1 Combined fluorescein leakage- resazurin reduction assay**

The deterioration of intercellular junctions (figure 1.04) may be demonstrated by the use of the fluorescein leakage assay (FLA) (Tchao, 1988; Shaw *et al.*, 1990; Zanvit *et al.*, 1999). The FLA was originally conceived by Tchao (1988) as an alternative to the Draize rabbit eye irritation assay and has since been used in a variety of forms (Tchao, 1988; Shaw *et al.*, 1990; Balls and Clothier, 1992; Martin and Stott, 1992). The rationale underlying the approach is the assumption that irritancy potential is related, in part, to the ability of the applied chemical to breach the epithelial barrier (Botham *et al.*, 1997). The barrier normally prevents the movement of substances from the exterior environment into the intercellular spaces between the epithelial cells and through to the deeper corneal layers, and is dependent on the existence of tight junctional complexes between the superficial cells of the epithelium (Tchao, 1988; Ward *et al.*, 1997b). The FLA is dependent upon two aspects of epithelial cell barrier function: tight junctions/desmosome and plasma membrane integrity (Tchao 1988; Botham *et al.*, 1997). Compromising either or both of these cellular structures through chemical or ionising radiation insult, causes an increase in permeability (Botham *et al.*, 1997). Several protocols have been published for the fluorescein leakage assay. In all cases either a monolayer or multilayer of epithelial cells is grown on a microporous membrane cell culture insert that provides a two-chamber



system (figure 1.17) to keep apical and basal culture media separated by the epithelial cell layer (Tchao, 1988; Shaw *et al.*, 1990; Balls and Clothier, 1992; Martin and Stott, 1992; Botham *et al.*, 1997; Ward *et al.*, 1997b).

**Figure 1.17** : A microporous membrane provides a two-chamber culture system, damage to the plasma membrane and/or interepithelial junctions is detected by fluorescein leaking through to the basal chamber.



Chemically induced loss of transepithelial impermeability may be determined by colorimetric measurements of the levels of fluorescein which have passed from the apical to the basolateral side of the mono- or multilayer (Tchao, 1988; Cottin and Zanvit, 1997; Zanvit *et al.*, 1999). If both the plasma membrane and epithelial junctions remain intact, only the limited fluorescein that has passed through the cells themselves is present in the basolateral chamber (figure 1.17). As the FLA is non-cytotoxic, it is possible to study both the ability of cells to recover from chemical

challenge, and the damage produced by chemicals that do not exhibit an immediate effect (Cottin and Zanvit 1997). Results are expressed as either fluorescein leakage as a percentage of that leaking through the membrane support alone. The FL20 or FL50 values, representing the dose of a test reagent causing either a 20% or 50% leakage, respectively, of fluorescein through the cell monolayer (Pitt *et al.*, 1987; Tchao, 1988; Shaw *et al.*, 1990; Balls and Clothier, 1992; Cook *et al.*, 1992; Martin and Stott, 1992; Botham *et al.*, 1997; Ward *et al.*, 1997a). The standard non-treated monolayer should show 6% or less leakage for the assay to qualify. Barrier function may also be measured by determining the transepithelial resistance (TER) of the epithelial sheet. This technique however, cannot distinguish between transcellular and paracellular permeability and alterations in cell volume as a consequent of chemical exposure may also significantly affect TER measurements whilst not significantly modulating barrier function. Although TER is routinely used to assess barrier integrity the assay measures ion flux in kidney models, neither water or solute flux are not and so is not necessarily an accurate measure of the barrier function (Ward *et al.*, 1995). The complexity of the procedure means that it is unsuitable for high throughput screening of potential irritants, although new multiple measuring devices are now available (Ryan *et al.*, 2005).

#### **1.14 Test Chemicals**

Test chemicals were selected upon the basis of their correlation with *in vivo* data. In addition, timolol maleate was selected as a clinically relevant pharmaceutical known to cause ocular irritancy as a result of prolonged use in the treatment of glaucoma (Baudouin *et al.*, 1998; Burgalassi *et al.*, 2000).

### 1.14.1 Sodium dodecyl sulphate

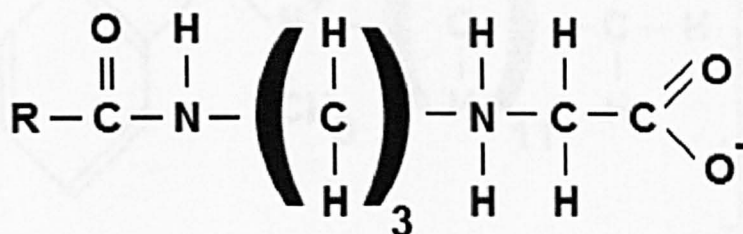
Anionic surfactants demonstrate a marked biological activity either by binding to various bioactive macromolecules such as starch (Merta and Stenius, 1999), proteins (Nielsen *et al.*, 2000), peptides and DNA (Marques *et al.*, 2000) or by inserting into various cell fragments (i.e. phospholipids membranes) causing malfunction (Cserhati *et al.*, 2002). Sodium dodecyl sulphate (SDS) (figure 1.18) is an important component of many dental cleansing products, emulsifying plaque deposits (Newbrun, 1983) and solubilising lipophilic substances whilst also acting as a foaming agent. In Europe and North America, its concentration in toothpastes ranges from 0.5% to 2.0% (Barkvoll and Rolla, 1994). It is well established that SDS is an irritant to skin, its application resulting in a dose-dependent contact dermatitis (Berardesca and Maibach, 1988; Ponc and Kempenaar, 1995). Its use in toothpaste may, by chronic exposure, damage the integrity of the oral mucosa (Herlofson and Barkvoll, 1996) and exaggerated use models have been shown to induce mucosal desquamation (Herlofson and Barkvoll, 1996). This indicates that prolonged exposure to SDS impairs the function of the oral mucosa, which as a result of similar morphology to that of the cornea, could apply to this tissue. Draize eye irritation studies reveal that sodium dodecyl sulphate is a moderate irritant with a MAS of 16 (Ohno *et al.*, 1999), and a MMAS of 16 at 3% , 59.2 at 15% and 60.5 at 30% (ECETOC Technical report 48 (2), 1998)

**Figure 1.18 :** Structural formulae of sodium dodecyl sulphate



Cocamidopropylbetaine (CAPB) (figure 1.20) is the most widely used amphoteric surfactant in the cosmetic industry (McFadden *et al.*, 2001) especially in shampoos and bath products. A number of studies have demonstrated that CAPB induces less degree of irritation both in skin and oral personal products compared to BAK and SDS (Herlofson and Barkvoll, 1996; Barany *et al.*, 1999). However, some studies indicate that CAPB in rare cases may be responsible for allergic reactions (Fowler, 1993; McFadden *et al.*, 2001) and this can be dependent on its purity (Foti *et al.*, 2003).

**Figure 1.20** : Structural formulae of cocamidopropylbetaine



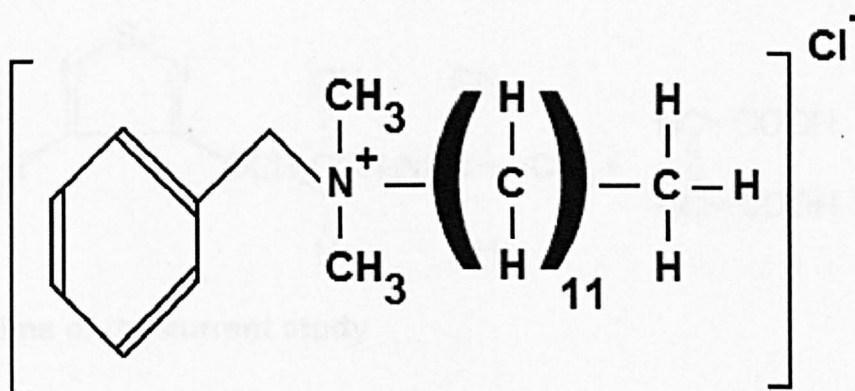
#### 1.14.4 Benzalkonium Chloride

Benzalkonium chloride (BAK) is a quaternary ammonium compound (figure 1.21) and is the most common antimicrobial preservative used in topical ophthalmic preparations (Berdy *et al.*, 1992; Pisella *et al.*, 2000). BAK is highly efficacious against numerous microbes and acts by denaturing proteins and causing the lysis of cytoplasmic membranes. The surfactant abilities of BAK enable it to solubilise the extracellular matrix (ECM) of the corneal epithelium, thereby, increasing the penetration of BAK (Gasset *et al.*, 1974; Kreine, 1979). BAK can accumulate in



ocular tissue (Cha *et al.*, 2004), remaining for relatively lengthy periods (Gasset *et al.*, 1974) and as a result, BAK can induce different types of cell death (De Saint Jean *et al.*, 2000) in a dose-dependent manner; apoptosis at very low concentrations (0.0001%) and necrosis at higher concentrations (0.05-0.1%) (De Saint Jean *et al.*, 2000). Draize eye irritation studies reveal that benzalkonium chloride is a severe irritant with a MAS of 78 (Ohno *et al.*, 1999) and a MMAS of 34.3 to 56.3 at 1%, 83.8 at 5% and 108 at 10% (ECETOC Technical report 48 (2), 1998),

**Figure 1.21** : Structural formulae of benzalkonium chloride

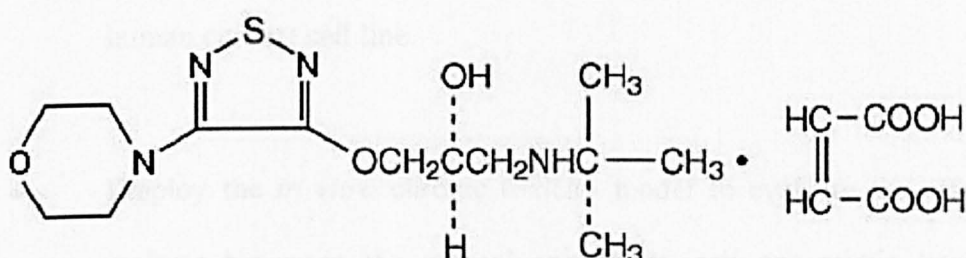


#### 1.14.5 Timolol Maleate

Topical  $\beta$ -adrenergic blockers are usually the first line of medical therapy prescribed to reduce the intraocular pressure in glaucoma and ocular hypertensive patients (Stewart *et al.*, 2000). Timolol maleate (figure 1.22) is a salt of an organic acid that has been demonstrated to have several types of cellular effects. First, it is used as an animal model in rats for the Fanconi syndrome, which is a renal disease affecting proximal renal tubule re-absorption of amino acids and electrolytes (Verani *et al.*, 1982). In addition, *in vitro* studies have demonstrated that maleate reduces

glutathione (GSH), a free radical scavenger, in both corneal epithelial and endothelial cells specifically. (Megaw, 1984; Riley, 1990; Stewart *et al.*, 2000). Prolonged use of timolol maleate (required for the treatment of glaucoma and ocular hypertension) is associated with irritation of both the cornea and conjunctiva (Stewart *et al.*, 2000). This may be the explanation to why many patients prescribed timolol maleate for glaucoma often discontinue their treatment compared to individuals prescribed alternatives (Baudouin *et al.*, 1998; Burgalassi *et al.*, 2000)

**Figure 1.22 :** Structural formula of timolol maleate



### 1.15 Aims of the current study

The main aims of the current study are;

- Evaluate the Japanese human corneal epithelial cell line (J-HCET) for use in an *in vitro* model of chronic ocular irritancy.
- Determine if a monolayer of J-HCET cells can correctly predict ocular irritancy of four representative surfactants using a range of biological endpoints when compared to the Draize eye irritation assay.

- Evaluate the effects of a subcytotoxic surfactant concentration over a 21 day exposure period in terms of viability and function subsequently to an acute surfactant exposure utilising the J-HCET cell line.
- Evaluate the effects growth medium composition has upon the differentiation and barrier function of the J-HCET cell line.
- To assess the use of commercially available animal product free growth supplement in conjunction with a defined culture medium, in terms of growth rate and modulation of responses to exogenously applied chemicals of a human corneal cell line.
- Employ the *in vitro* chronic toxicity model to evaluate the effects timolol maleate has upon the corneal epithelium, can one mimic known *in vivo* corneal epithelial adverse affects *in vitro*?



## **Chapter 2**

### ***Materials and Methods***

# Materials & Methods

The procedures commonly used during this study have been described in this chapter. Techniques specific to particular investigations are described in the appropriate chapters.

## 2.1 MATERIALS

### 2.1.1 Chemicals

Heat inactivated foetal bovine serum (FBS) was obtained from Autogene Bioclear (Wiltshire, U.K). Ammonium sulphamate, benzalkonium chloride (BAK), buthionine sulfoxamine (BSO), cocamidopropylbetaine (CAPB), dimethylsulphoxide (DMSO), ethanol, glacial acetic acid, Hanks balance salt solution (HBSS with calcium and magnesium), hydrochloric acid, L-glutathione (GSH), mercury (II) chloride, methanol, n-(1-naphthyl)ethylenediamine dihydrochloride, neutral red (CI50040), paraformaldehyde, penicillin (10000 IU ml<sup>-1</sup>) /streptomycin (10mgml<sup>-1</sup>) solution in 0.8% sodium chloride, fluorescein isothiocyanate (FITC) labeled phalloidin, resazurin, resorufin, sodium bicarbonate, sodium fluorescein, sodium nitrite, sodium dodecyl sulphate (SDS), sulphanilamide, sulphosalicylic acid dehydrate, triton X-100, Trypsin/EDTA (porcine, 1mgml<sup>-1</sup> in 0.4mgml<sup>-1</sup> EDTA) Coomassie®brilliant blue R 250, milling blue 2BR (Kenacid Blue) and tween 20 (T20) was obtained from Sigma-Aldrich Co Ltd (Poole, U.K). Phosphate buffered saline tablets (with calcium and magnesium) from Oxoid (Basingstoke, UK). Anhydrous calcium chloride and citric acid obtained from

Fischer Scientific Ltd (Loughborough, UK). Carbon dioxide and liquid nitrogen obtained from British Oxygen Company (BOC) (Surrey, UK). Epilife keratinocyte calcium free growth media, collagen coating kit (containing an undisclosed concentration of human recombinant type I collagen), human keratinocyte growth supplements (HKGS) and human keratinocyte growth supplements V2 (HKGS-V2) from Cascade Biologics (Mansfield, U.K). KBM keratinocyte growth media and KGM Singlequots™ from Cambrex Bioscience Wokingham, Ltd (Wokingham, UK). Fibronectin-collagen (FNC) (containing 10µgml<sup>-1</sup> bovine fibronectin, 35µgml<sup>-1</sup> bovine type I collagen) coating kit was obtained from AthenaES (Baltimore, USA). Haychlor Industrial, Trigene II and liquid detergent ES®7X was purchased from Scientific Laboratory Supplies (Nottingham, UK). Transmit™ resin was obtained from TAAB laboratories Equipment Ltd (Berkshire, UK). Chemicals were stored as recommended by the manufacturer.

### **2.1.2 Consumables**

Bijoux (7ml), and 30ml universal bottles were purchased from Bibby Sterilin Ltd (Staffordshire, UK). Corning Costar disposable serological pipettes (5ml, 10ml, 25ml) and disposable reservoir liners (8 channel) were purchased from Corning Incorporated (Loughborough, UK). Autoclave bags, indicator tape, yellow biohazard bags and sterile plastic packaging were obtained from NHS supplies. Cryopreservation vials and pipette tips (200µl and 1000µl) were purchased from Starstedt (Leicester, UK). Biological hazard tape was purchased from Sellotape. Hand towels were obtained from Kimberley-Clark® Corporation. Cellstar tubes (50ml) were purchased from Greiner Labortechnik (Poitiers, France). Long form

unplugged glass pasteur pipettes were purchased from Mexcel. Minisart high flow aerodisc filters (pore size 0.2 $\mu$ m) were purchased from Sartorius A.G (Goettingen, Germany). Powder free non-sterile vinyl gloves were obtained from Labsales Co (Cambridge, UK). Universal graduated glass bottles, chamber slides (8 well), coverslips, culture flasks (125cm<sup>2</sup>, 80cm<sup>2</sup> and 25cm<sup>2</sup>), 24 and 96 well flat base culture plates and polycarbonate (10mm diameter, 0.4 $\mu$ m pore size) culture inserts were purchased from Scientific Laboratory Supplies (suppliers of Nunc Brand Products) (Nottingham, UK).

### **2.1.3 Equipment**

Medical Centaur 2 MSE centrifuge obtained from Thermolife Sciences (Paisley, UK). LEEC MKII proportional temperature controlled CO<sub>2</sub> incubator was purchased from LEEC (Cardiff, UK). Laboratory microscope obtained from Nikon (Japan). Confocal microscope TCS4D obtained from Leica (Heidelberg, Germany). Power pipette gun purchased from Jencons-PLS (Leighton Buzzard, UK). Waterbaths (models WB14 and Y14) obtained from CamLab Ltd (Cambridge, UK). Class II culture cabinets (ICN Biomedicals and MDH) were purchased from Walker Safety Cabinets (Derby, UK). FLUORstar Galaxy plate reader obtained from BMG, Lab Technologies (Buckinghamshire, UK). Analytical balance, ASYS HITEC Expert 96 plate reader, Mr. Frosty™, surgical forceps, Neubaur improved haemocytometer, pipettes (50-300 $\mu$ l) and scalpels obtained from Scientific Laboratory Supplies (Nottingham, UK). Dymax 30 aspirator vacuum pumps obtained from Charles Austen Pumps. Autoclave obtained from Denley (Colchester, UK). Rotatest plate shaker was purchased from Luckham Ltd (Essex, UK). Microscope slides and 22 x

64mm glass coverslips obtained from Chance Proper Ltd (West Midlands, UK). IBM compatible PC running Microsoft Windows XP™ Professional, Microsoft Office XP™ Professional and GraphPad Prism 4.0 was purchased from Fujitsu-Siemens (Nottingham, UK).

**2.1.4 Immunostaining reagents**

Vectashield™ mounting medium containing propidium iodide (50µgml<sup>-1</sup>) was obtained from Vector Laboratories (California, U.S.A). Normal goat serum and bovine serum albumin (BSA; heat and alcohol fractionated) obtained from Sigma-Aldrich Company Ltd (Poole, U.K). Phosphate buffered saline (PBS) (calcium- and magnesium- free) was prepared, as directed by the manufacturer from pre-weighed tablets added to distilled water, autoclaved in 500ml or 50ml aliquots (as required) and stored at room temperature. Primary and secondary antibodies were obtained as outlined in tables 2.0 and 2.1 respectively.

**Table 2.0 : Primary antibodies used for immunochemical staining.**

Antigen Specificity	Host	Form	Reactivity	Working Dilution	Source
Claudin-1 (C Terminus)	Rabbit	Polyclonal	Human	25µgml <sup>-1</sup>	Zymed (San Fransisco, USA)
E-Cadherin (C Terminus)	Mouse	Monoclonal	Human, Dog, Rat, Mouse	25µgml <sup>-1</sup>	BD Biosciences (Oxford, UK)
Occludin (C Terminus)	Mouse	Monoclonal	Human, Mouse, Rat, Dog	10µgml <sup>-1</sup>	BD Biosciences (Oxford, UK)
Occludin (C Terminus)	Rabbit	Polyclonal	Human, Mouse, Dog, Rat	10µgml <sup>-1</sup>	Zymed (San Fransisco, USA)
ZO-1 (C Terminus)	Mouse	Monoclonal	Human, Dog	10µgml <sup>-1</sup>	Zymed (San Fransisco, USA)

**Table 2.1 : Secondary antibodies used during immunostaining.**

Antigen Specificity	Host	Form	Reactivity	Label	Working Dilution	Source
Rabbit IgG	Goat	Monoclonal	Rabbit Fc	FITC	25µgml <sup>-1</sup>	Sigma-Aldrich (Poole, UK)
Mouse IgG	Goat	Monoclonal	Mouse Fc	FITC	25µgml <sup>-1</sup>	Sigma-Aldrich (Poole, UK)

Antibodies were aliquoted into cryovials and stored and -20<sup>0</sup>C until required.

**2.1.5 Immortalised human corneal epithelial (J-HCET) cell line**

The SV40 transfected human corneal epithelial cell line (J-HCET) was a kind gift from Professor Araki-Sasaki (Institute of Ophthalmology, Tane Memorial Eye Hospital, Sakaigawa, Osaka, Japan).

**2.1.6 Reagents and stock solutions**

**2.1.6.1 Fluorescein leakage assay**

**a.) Resazurin solution**

Resazurin stock (5ml, 1mgml<sup>-1</sup> in HBSS) was filter sterilized into a 50ml universal container. HBSS (45ml) with calcium (as required) and magnesium was added to create a stock dilution of 100µgml<sup>-1</sup>. The resazurin stock was subsequently diluted 1:10 in HBSS and wrapped in aluminium foil to prevent photoreduction. Resazurin

solution ( $10\mu\text{gml}^{-1}$ ) was stored at  $4^{\circ}\text{C}$  and used within 2 weeks. Where possible, for a given series of experiments the same prepared resazurin solution was employed.

#### **b.) Resazurin- sodium fluorescein (R/SF) solution**

Sodium fluorescein (0.05g) was completely dissolved in 500ml of  $10\mu\text{gml}^{-1}$  resazurin solution (section 2.1.6.5) to give a final concentration of 0.01% fluorescein in  $10\mu\text{gml}^{-1}$  resazurin. R/SF solution was wrapped in aluminium foil to prevent photoreduction, stored at  $4^{\circ}\text{C}$  and used within 4 weeks. Where possible, for a given series of experiments the same R/SF solution was employed.

### **2.6.1.2 Glutathione equivalents assay**

#### **a.) Acidified mercury (II) chloride - sulphanilamide solution**

Mercury (II) chloride (0.2g) and sulphanilamide (0.68g) were completely dissolved in 100ml 0.32M hydrochloric acid (HCl) and stored at  $4^{\circ}\text{C}$ .

#### **b.) Acidified n-1-naphthylethylenediamine solution (0.1% w/v)**

N-1-naphthylethylenediamine (10mg) was completely dissolved in 10ml of 0.4M hydrochloric acid (HCl), immediately prior to use.

**c.) Ammonium sulphamate (0.5% w/v)**

Ammonium sulphamate (1g) was completely dissolved in 200ml of distilled water. The solution was stored at 4°C.

**d.) Acidified sodium nitrite (1mM) solution**

Sodium Nitrite ( $\text{NaNO}_2$ ) (1mM) was dissolved in 0.5M hydrochloric acid (HCl) and stored at 4°C.

**e.) Glutathione (1mM) solution**

Glutathione (3.07mg) was completely dissolved in 10ml of aqueous sulphosalicylic acid (5% w/v).

**f.) Hydrochloric acid (0.5, 0.4 & 0.32M)**

Hydrochloric acid (HCl) (1M) stock was produced by dilution of concentrated acid with distilled water ( $\text{dH}_2\text{O}$ ). Stock solution was diluted using distilled water to produce 0.5M, 0.4M and 0.32M working dilutions. Working dilutions were stored at room temperature.



### **g.) Sulphosalicylic acid (5% w/v)**

Sulphosalicylic acid (5g) was completely dissolved in 100ml distilled water and stored at 4°C in the dark.

### **2.1.6.3 Kenacid blue (total protein) assay**

#### **a.) Kenacid Blue fixative solution.**

Neutral red desorb solution was made by the addition of 10ml glacial acetic acid and 490ml distilled water to 500ml ethanol. The solution was stored at room temperature until required.

#### **b.) Kenacid blue staining solution**

Kenacid Blue (KB) stock solution was prepared by adding 0.4g Coomassie® brilliant blue R 250 (Kenacid Blue) to 250ml ethanol and 630ml distilled water. The solution was mixed thoroughly and stored at room temperature. Kenacid Blue working solution was prepared by addition of 12ml glacial acetic acid to 88ml Kenacid Blue stock solution immediately prior to use.

### **c.) Kenacid blue wash solution**

Washing solution was prepared by the addition of 100ml ethanol and 50ml glacial acetic acid to 850ml distilled water. The solution was stored at room temperature until required.

### **d.) Kenacid Blue desorb solution**

Kenacid Blue desorb solution was prepared by completely dissolving 98.15g potassium acetate (equivalent to 1M) to 700ml ethanol and 300ml distilled water. The solution was stored at room temperature.

## **2.1.6.4 Imaging techniques**

### **a.) Normal goat serum (NGS) (5% v/v)**

Heat inactivated normal goat serum (500 $\mu$ l) was added to 9.5ml phosphate buffered saline immediately prior to use.

### **b.) Paraformaldehyde solution (4% w/v)**

Paraformaldehyde (4g) was added to 100ml PBS and heated to 60°C with continuous agitation until completely dissolved. The solution was filtered and stored at -20°C until required.

### **c.) Triton X-100 solution (0.1% v/v)**

Triton X-100 (10 $\mu$ l) was suspended in 9.99ml phosphate buffered saline (PBS) immediately prior to use.

## **2.1.7 Preparation of Epilife cell culture media**

### **2.1.7.1 Cascade Biologics Epilife™ culture media**

Cascade Biologics Epilife™ growth media (500ml) was prepared as directed by the manufacturer with either HKGS [bovine pituitary extract (0.2% v/v), bovine insulin (5 $\mu$ gml<sup>-1</sup>), hydrocortisone (0.18 $\mu$ gml<sup>-1</sup>), bovine transferrin (5 $\mu$ gml<sup>-1</sup>), human epidermal growth factor (0.2ngml<sup>-1</sup>)] or HKGS-V2 [human recombinant insulin-like growth factor type-1, prostaglandin E-2, human recombinant epidermal growth factor, hydrocortisone and a proprietary plant extract at undisclosed concentrations] supplements, penicillin (10,000 IU ml<sup>-1</sup>) streptomycin (10mgml<sup>-1</sup>) solution (5ml) and calcium chloride to give a final concentration of 1mM calcium in 500ml of complete medium. Anhydrous calcium chloride (4.41g) was dissolved in 100ml distilled water and filter sterilized. The solution was stored at 4°C and used within 1 month. Medium was stored at 4°C in the dark and used within 4 weeks of preparation.

### 2.1.7.2 Chronic surfactant exposure culture media

An appropriate mass of test surfactant (SDS, T20, CAPB or BAK), was suspended in cell culture medium to create concentrations as outlined in table 2.2. Medium was filter sterilised, stored at 4°C and used within 4 weeks.

**Table 2.2 :** Chronic surfactant exposure cell culture media concentrations.

Surfactant	Concentration ( $\mu\text{gml}^{-1}$ )
Tween 20	25
Sodium dodecyl sulphate	4
Cocamidopropyl bentaine	3
Benzalkonium chloride	0.0025

### 2.1.7.3 Chronic surfactant exposure culture media with neutral red dye

Immediately prior to use neutral red dye ( $3.3\text{mgml}^{-1}$ ; Sigma, Poole, UK) ( $125\mu\text{l}$ ) was added to 11ml media to give a final concentration of  $37.5\mu\text{gml}^{-1}$  and filter sterilized. This was kept at 37°C and used within 1 hour of preparation to avoid problems associated with neutral red crystals, that can occasional occur.

### 2.1.7.4 Culture media quality control

Prior to use, a bijoux containing 5ml of prepared media (sections 2.1.7.1/2) was incubated for 2-3 days at 37°C, 5% (v/v) CO<sub>2</sub> in air and inspected microscopically for micro-organism contamination. If contamination was present, the media was

discarded.

## **2.2 METHODS**

### **2.2.1 Sterilization**

#### **2.2.1.1 Autoclaving**

Sterilization by autoclaving was carried out in an autoclave at 15lb/sq inch for 20 minutes at 120°C. Autoclave indicator tape was employed to confirm heating.

#### **2.2.1.2 Glassware**

All items of glassware were washed in liquid detergent ES®7X, rinsed in distilled de-ionized water, air dried and autoclaved (section 2.2.1.1).

### **2.2.2 Microscopy**

#### **2.2.2.1 Light Microscopy**

All microscopic observations were carried out using a Nikon inverted microscope with 10 times eye piece, 2.5, 4 or 10 times objective.

### **2.2.2.2 Confocal Microscopy**

Fluorescein isothiocyanate (FITC) immunofluorescence was detected using a Leica TCS4D confocal microscope with an excitation filter of 485nm, and an emission of 530nm.

### **2.2.3 Routine maintenance of J-HCET cell line**

#### **2.2.3.1 Culture conditions**

All experimental work and tissue culture manipulations were conducted in a sterile class 2 laminar flow cabinet using sterile techniques. When required both plasticware and glassware were coated using coating kits as directed by the manufacturer.

#### **2.2.3.2 Calculation of cell number**

A haemocytometer was used to determine the number of cells using a light microscope. Cells that crossed the triple line at the side and bottom of the square were excluded from the count. The volume of cell suspension required to give the required seeding density was then calculated using equation 2.0.

**Equation 2.0 :** Determining the volume of cell suspension and media required for the appropriate experimental seeding density.

$$V_1 = \frac{(C_2) (V_2)}{(C_1)}$$

*Where  $V_1$  denotes total ml of cell stock required,  $C_1$  denotes total number of cells in stock,  $V_2$  denotes final volume of cell suspension needed and  $C_2$  is final cell suspension concentration.*

### **2.2.3.3 Serial propagation of J-HCET cell line**

Growth medium was warmed to 37°C immediately prior to cell harvesting. Medium was aspirated from large (125cm<sup>2</sup>) culture flasks, and J-HCET cells washed with 20ml of sterile PBS (pre-warmed to 37°C). PBS was aspirated and a second wash performed. The cells were incubated with 10ml EDTA/trypsin for 5 minutes at 37°C, 5% (v/v) CO<sub>2</sub> in air. Foetal bovine serum (FBS) (10ml, 10% v/v in PBS) was applied to stop the action of the trypsin and the suspension transferred to sterile 20ml containers and centrifuged at 1000 rpm for 5 minutes at room temperature. The supernatant was aspirated and the cellular pellet re-suspended in 10ml of cell culture medium. A sample of the the cell suspension was counted using a haemocytometer, and a large culture flask seeded with 5x10<sup>5</sup> cells in 20ml of medium i.e. 2.5 x 10<sup>4</sup> cell ml<sup>-1</sup>. Cultures were maintained at 37°C, 5% (v/v) CO<sub>2</sub> in air, re-fed with fresh culture medium every 72 hours and sub-cultured once cells were 70-80% confluent. Cells were monitored on a daily basis at low magnification (x10 objective) to

ascertain the level of cell growth, the morphology of the cells and check that micro-organism contamination had not occurred.

#### **2.2.3.4 Cryopreservation of J-HCET cell line**

J-HCET were harvested, pelleted and re-suspended in FBS containing 10% dimethylsulphoxide (DMSO). Cryovials were seeded at a density of  $1 \times 10^6$  cells per ml and frozen to  $-80^{\circ}\text{C}$  over 24 hours in Mr. Frosty™. Cryovials were placed in liquid nitrogen for long term storage.

#### **2.2.3.5 Retrieval of cryopreserved J-HCET cell line**

Growth media were warmed to  $37^{\circ}\text{C}$  immediately prior to cell retrieval. Cryovials were retrieved from liquid nitrogen storage and rapidly thawed to room temperature. The contents of the vial were transferred to 9ml of medium and centrifuged for 5 minutes at 1000rpm. The supernatant was aspirated and the pellet resuspended in 10ml medium. Large ( $125\text{cm}^2$ ) flasks were seeded with the 10mls of suspension, and maintained at  $37^{\circ}\text{C}$ , 5% (v/v)  $\text{CO}_2$  in air, re-fed with fresh culture medium every 72 hours and sub-cultured once cells were 70-80% confluent.

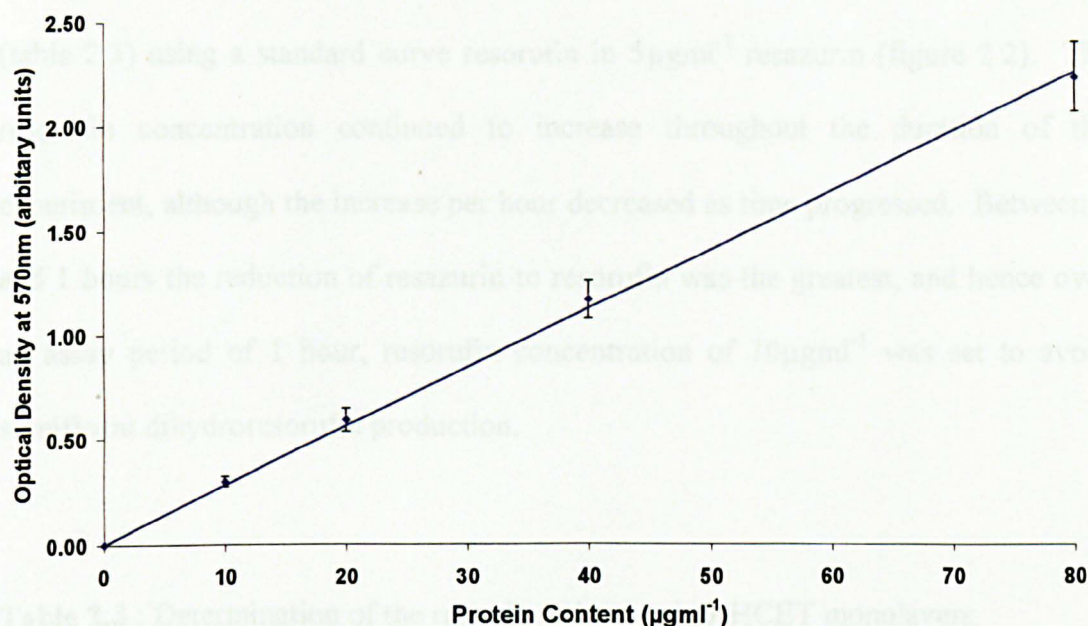


## **2.2.4 Cytotoxicity assays**

### **2.2.4.1 Kenacid blue total protein assay**

The kenacid blue protein assay was used to determine the total protein content in the fixed cells in wells containing monolayers, by the previously described method routinely used in the laboratory (INVITTOX protocol 3B; Knox *et al.*, 1986; Clothier, 1995). The Kenacid blue fixative solution (200 $\mu$ l) was added and allowed to evaporate over 48 hours at 37°C. The acidified kenacid blue solution (100 $\mu$ l) was applied. Plates were agitated on a rotatest shaker for 2 hours. Wells were aspirated and unbound dye removed by washing twice with kenacid blue wash solution for 20 minutes. Kenacid blue desorb (section 2.1.6.3), 100 $\mu$ l was added and the plates agitated for 60 minutes. The optical density of each well was then read using an ASYS HITECH Expert plate reader at 570 $\pm$ 10nm. A standard protein curve was produced using concentrations of BSA (0-160 $\mu$ gml<sup>-1</sup>) in 70% ethanol (Gray *et al.*, 2004). Total cell protein was calculated from the concentration curve which gave a linear increase in optical with increasing standard protein concentrations (figure 2.0). Total protein was expressed as  $\mu$ gml<sup>-1</sup> of protein.

**Figure 2.0 :** Kenacid blue assay standard curve (mean±S.D, n=12).



For linear section;  $y=0.0283x$  where  $y$  is absorbance at 570nm and  $x$  is protein content ( $\mu\text{gml}^{-1}$ ). At protein concentrations above  $80\mu\text{gml}^{-1}$  the standard curves exhibits non-linear behaviour.

## 2.2.4.2 Resazurin reduction assay

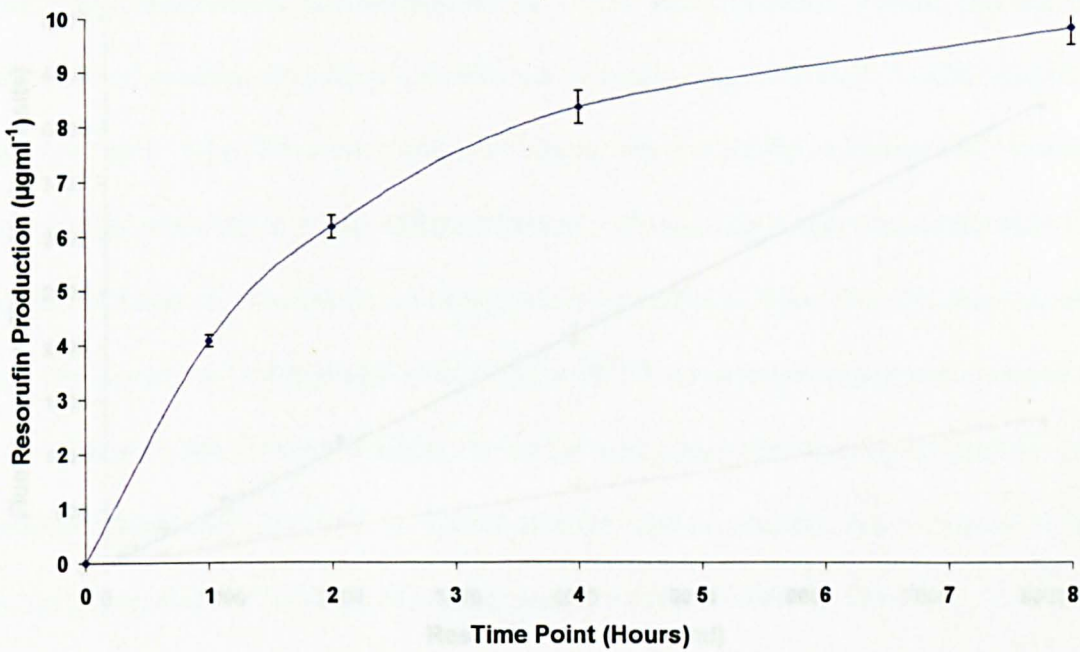
Resazurin is a tetrazolium-based dye (O'Brien *et al.*, 2000), incorporating resazurin and resorufin as oxidation-reduction indicators that yield colorimetric changes and a fluorescent signal (when excited at a specific wavelength of 530nm) in response to cell metabolic activity. In an irreversible reaction, the blue non-fluorescent oxidised form (resazurin) becomes pink and fluorescent (resorufin) upon reduction (Andrew *et al.*, 1997; O'Brien *et al.*, 2000; Byth *et al.*, 2001). The possibility that the resorufin

produced would be further reduced to dihydroresorufin was minimized by using resazurin in excess ( $10\mu\text{gml}^{-1}$  in HBSS). Measurements were made on J-HCET cultures at 1, 2, 4, and 8 hour intervals (figure 2.1), to determine production ( $\mu\text{gml}^{-1}$ ) (table 2.3) using a standard curve resorufin in  $5\mu\text{gml}^{-1}$  resazurin (figure 2.2). The resorufin concentration continued to increase throughout the duration of the experiment, although the increase per hour decreased as time progressed. Between 0 and 1 hours the reduction of resazurin to resorufin was the greatest, and hence over an assay period of 1 hour, resorufin concentration of  $10\mu\text{gml}^{-1}$  was set to avoid significant dihydroresorufin production.

**Table 2.3 :** Determination of the rate of production in J-HCET monolayers.

Time Point (Hours)	Rate of Resorufin Production ( $\mu\text{gml}^{-1}\text{h}^{-1}$ )
0 - 1	4
1 - 2	2
2 - 4	1
4 - 8	0.37

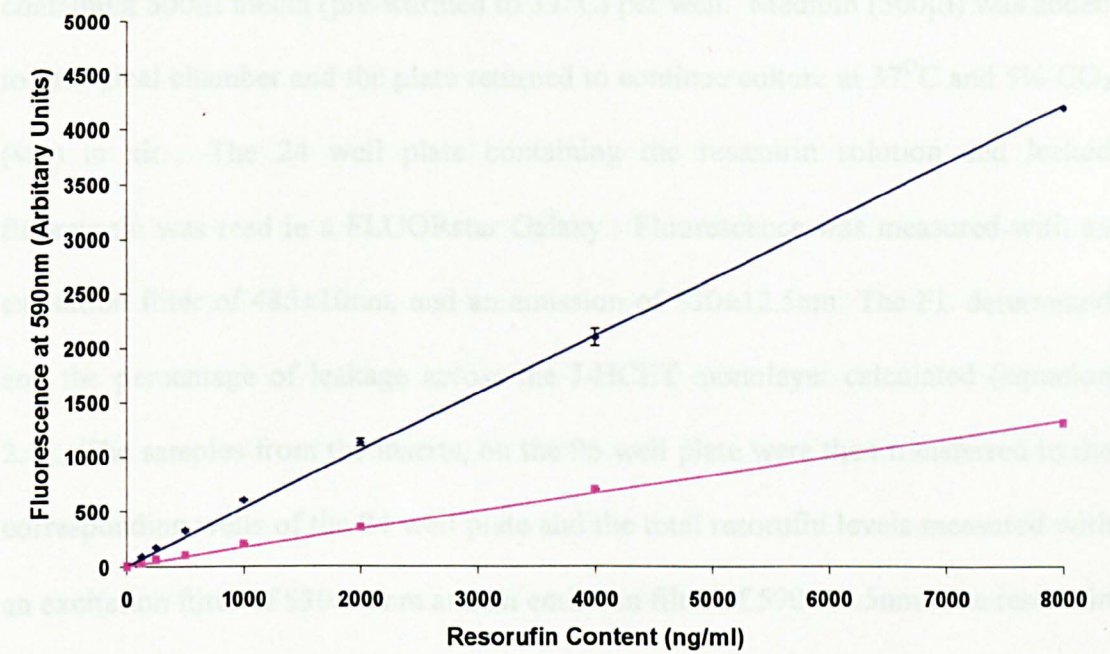
**Figure 2.1 :** Resorufin production over 8 hours (mean±S.D, n=3).



Resazurin solution (section 2.1.6.4) was pre-warmed to 37°C. Medium was aspirated from the wells and 200µl resazurin solution applied. Plates were incubated at 37°C, 5% (v/v) CO<sub>2</sub> in air for 55 minutes. Plates were read at 60 minutes using the FLUORstar Galaxy with an excitation filter of 530±10nm and an emission filter of 590±12.5nm. Resazurin solution was aspirated and medium pre-warmed to 37°C, added. Plates were returned to the incubator 37°C, 5% (v/v) CO<sub>2</sub> in air for 24 hours. Standard curves were produced using samples of 0, 125, 250, 500, 1000, 2000, 4000, 8000 and 16000ngml<sup>-1</sup> resorufin in 5µgml<sup>-1</sup> resazurin in HBSS (figure 2.2). The standard curve was used to determine the amount of resorufin in each sample from their respective absorbencies.



**Figure 2.2 : Resazurin reduction assay standard curves (mean±S.D, n=3).**



For linear section; ■ 96 well plate  $y=0.528x$ , ■ 24 well plates (used with fluorescein leakage assay)  $y=0.168x$  where  $y$  is emission at  $590\pm12.5\text{nm}$  and  $x$  is resorufin concentration ( $\text{ngml}^{-1}$ ).

**2.2.4.3 Combined fluorescein leakage- resazurin reduction assay**

At all times during the protocol the inserts were handled with sterile forceps. Medium was aspirated from the inserts which were subsequently transferred to a fresh 24 well plate, containing 400µl of resazurin solution per well (pre-warmed to 37°C). Resazurin/sodium fluorescein (0.01%) solution (2.1.6.1) (200µl) was added to the insert, and the plate incubated for 90 minutes at 37°C and 5% CO<sub>2</sub> (v/v) in air. After 85 minutes, the plate was removed from the incubator in preparation for the

removal of the RS/FL samples from each insert. The samples from the inserts were placed in a 96 well plate. The inserts were transferred to a fresh 24 well plate containing 500µl media (pre-warmed to 37°C) per well. Medium (500µl) was added to the apical chamber and the plate returned to continue culture at 37°C and 5% CO<sub>2</sub> (v/v) in air. The 24 well plate containing the resazurin solution and leaked fluorescein was read in a FLUORstar Galaxy. Fluorescence was measured with an excitation filter of 485±10nm, and an emission of 530±12.5nm. The FL determined and the percentage of leakage across the J-HCET monolayer calculated (equation 2.1). The samples from the inserts, on the 96 well plate were then transferred to the corresponding wells of the 24 well plate and the total resorufin levels measured with an excitation filter of 530±10nm and an emission filter of 590±12.5nm. The resorufin standard curve (24 well plate; figure 2.2) was used to determine the conversion of resazurin to resorufin in each sample from their respective absorbencies.

**Equation 2.1** : Determining the degree of leakage across the J-HCET monolayer

$$\left( \frac{\text{Fluorescein that crossed the monolayer}}{\text{Fluorescein that crossed the no-cell insert}} \right) \times 100$$

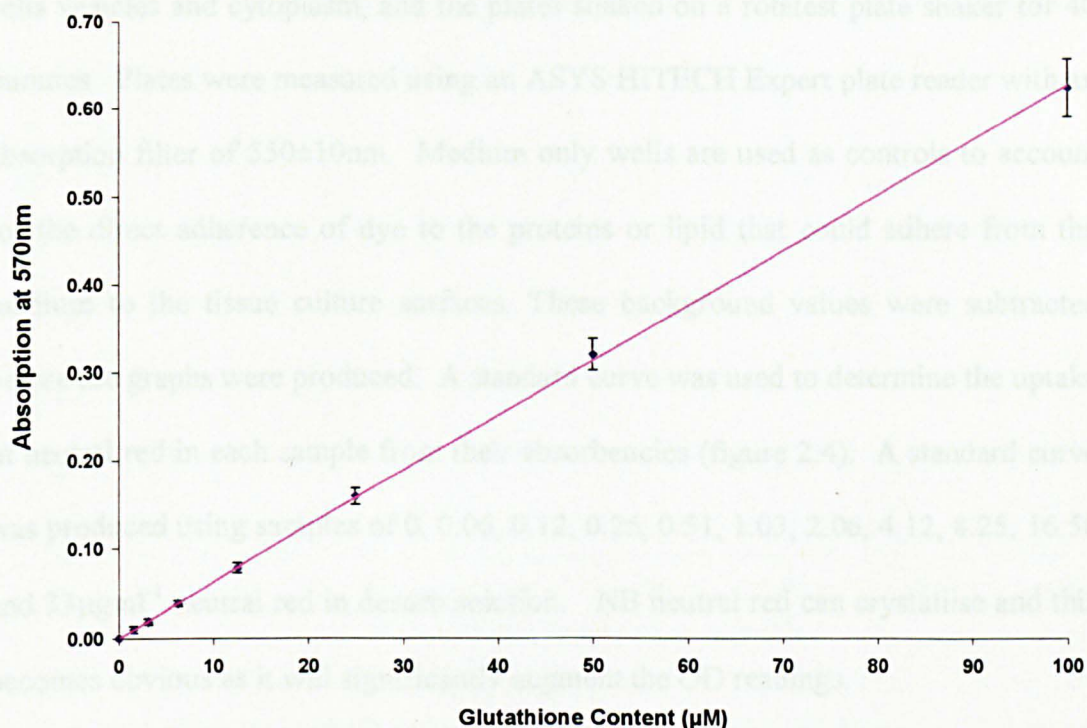
#### 2.2.4.4 Glutathione equivalents assay (GSH<sub>EQ</sub>)

The colorimetric GSH assay was used to determine the amount of thiols within the J-HCET monolayers. The method was adapted from Saville (1958) for use in 96 well plates. The assay worked on the assumption that the majority of thiols within the J-HCET were those in the GSH molecules. Wells were aspirated and washed with

200µl HBSS. A 20µM glutathione standard (50µl) was run with each experiment to ensure consistency. Sulphosalicylic acid solution (50µl) was added to the wells and the plates agitated for 30 minutes on a rotatest plate shaker. Sodium nitrate (1mM, 50µl) was added to each well and agitated on a rotatest plate shaker for 5 minutes. Aqueous ammonium sulphamate (20µl, 0.5% w/v) was added to each well and the plate agitated for 15 minutes. Mercury (II) chloride (0.2% w/v) and sulphanilamide (0.68% w/v) solution, 65µl was added to each well and immediately 50µl, 0.1% (w/v) n-1-naphthylethylenediamine dihydrochloride solution was added. The colour of the solution was allowed to develop for 5 minutes. Samples (200µl) were transferred to the corresponding position in a 96 well plate and the absorbance measured using an ASYS HITECH Expert plate reader at 550±10nm. Glutathione (1mM) standard solution was used to create dilutions of 0, 1.56, 3.13, 6.25, 12.5, 25, 50 and 100µM GSH in sulphosalicylic acid (5% v/v in dH<sub>2</sub>O). Dilutions (50µl) were added to the wells of a 96 well plate and the glutathione equivalents (GSH<sub>EQ</sub>) assay performed. Samples (200µl) were transferred to the corresponding position of a 96 well plate and the absorbance measured at 550±10nm. Absorbance readings were collated to produce a standard curve (figure 2.3).



**Figure 2.3 :** Glutathione equivalents assay standard curve (mean $\pm$ S.D, n=9).



For linear section;  $y=0.0063x$  (where  $y$  is absorption at  $550\pm 12.5\text{nm}$  and  $x$  is glutathione content ( $\mu\text{M}$ )). At glutathione concentrations above  $100\mu\text{M}$  the standard curves exhibits non-linear behaviour.

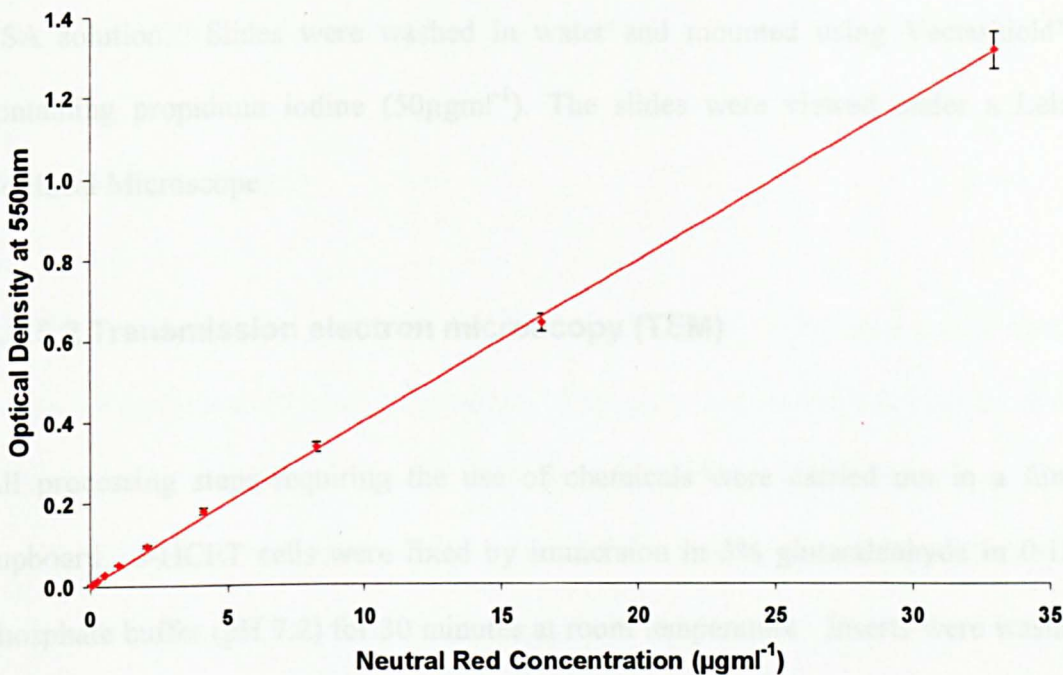
#### 2.2.4.5 Neutral red uptake (NRU) assay

The neutral red uptake assay measures the uptake of the neutral red (vital dye) compound by cells and is a simple and effective measure of cell viability as used in the validated 3T3 phototoxicity study (Spielmann *et al.*, 1998; Lovschall *et al.*, 2002). Media was aspirated from the wells and the monolayers washed twice with  $200\mu\text{l}$  HBSS (containing  $1\text{mM}$  calcium). Medium containing neutral red dye ( $33\mu\text{gml}^{-1}$ ) was added ( $250\mu\text{l}$ ) and the plate incubated for 3 hours at  $37^{\circ}\text{C}$ ,  $5\%$   $\text{CO}_2$  (v/v) in air. The neutral red containing medium was aspirated and the cells washed



twice with HBSS to remove unbound dye. Neutral red desorb solution (the kenacid blue fixative solution; section 2.1.6.3a) was added (100 $\mu$ l) to extract the dye in the cells vesicles and cytoplasm, and the plates shaken on a rotatest plate shaker for 40 minutes. Plates were measured using an ASYS HITECH Expert plate reader with an absorption filter of 550 $\pm$ 10nm. Medium only wells are used as controls to account for the direct adherence of dye to the proteins or lipid that could adhere from the medium to the tissue culture surfaces. These background values were subtracted before the graphs were produced. A standard curve was used to determine the uptake of neutral red in each sample from their absorbencies (figure 2.4). A standard curve was produced using samples of 0, 0.06, 0.12, 0.25, 0.51, 1.03, 2.06, 4.12, 8.25, 16.50 and 33 $\mu$ gml<sup>-1</sup> neutral red in desorb solution. NB neutral red can crystallise and this becomes obvious as it will significantly augment the OD readings.

**Figure 2.4 :** Neutral red assay standard curve (mean±S.D, n=4).



For linear section;  $y=0.0398x$  (where  $y$  is absorption at  $550\pm12.5\text{nm}$  and  $x$  is neutral red dye content ( $\text{mgml}^{-1}$ )).

**2.2.5 Imaging techniques**

**2.2.5.1 Indirect immunohistochemistry**

Cultures were fixed in paraformaldehyde solution (1% w/v in PBS) for 30 minutes and then 200µl Triton X-100 (0.1% v/v in PBS) for 5 minutes to permeabilize the cells. Normal goat serum (NGS) (5% v/v in PBS) was added for 30 minutes at room temperature to block non-specific binding of the secondary antibody. The primary antibody (table 2.0) was diluted in 0.1% BSA (w/v) in PBS and incubated at 4°C overnight. Monolayers were rinsed with BSA (0.1% w/v in PBS) solution and washed in fresh BSA solution. The secondary antibody (table 2.1) was diluted in

(0.1% BSA w/v in PBS), applied and incubated at room temperature for 1 hour in the dark. Slides were rinsed with BSA (0.1% w/v in PBS) solution and washed in fresh BSA solution. Slides were washed in water and mounted using Vectashield™ containing propidium iodine ( $50\mu\text{gml}^{-1}$ ). The slides were viewed under a Leica Confocal Microscope.

#### **2.2.5.2 Transmission electron microscopy (TEM)**

All processing steps requiring the use of chemicals were carried out in a fume cupboard. J-HCET cells were fixed by immersion in 3% glutaraldehyde in 0.1M phosphate buffer (pH 7.2) for 30 minutes at room temperature. Inserts were washed three times in 0.1M phosphate buffer and stored in 6% sucrose in 0.1M phosphate buffer at 4°C until required. This fixation does not add electron opacity to the J-HCET, so staining and post fixation in osmium tetroxide ( $\text{OsO}_4$ ), uranyl acetate and lead citrate for 30 minutes. This step is required when ultrathin sections are to be examined. The inserts were washed five times in distilled water before immersion in a 2% (w/v in distilled water) solution of uranyl acetate for 30 minutes protected from light. The fixed cells were dehydrated through graded alcohols before infiltration with resin. During the dehydration steps the polycarbonate membrane was removed from the polystyrene casing of the culture insert and further processing carried out on the cells attached to the membrane alone. Transmit™, a low viscosity low toxicity resin was used for these delicate samples. Once infiltrated with the resin/hardener/accelerator mixture, the blocks were moved to an oven and polymerised overnight at 70°C. Sections of 1µm were taken to orientate the blocks for ultrathin sections of a suitable portion of the monolayer. Ultrathin sections (70-

90nm) were cut on a Reichert Ultracut E microtome, placed on copper grids and contrasted with Reynolds lead citrate. Samples were then viewed in a Phillips 410 Transmission Electron Microscope operated at 80KV.

### **2.2.6 Analysis of data**

All experiments were performed at least in triplicate, with intra-experimental replicates performed for each experiment. The mean and standard deviations (S.D) were determined using raw data in Microsoft Excel XP. When repeat runs were available the repeat measure ANOVA (analysis of variance) test was employed with the post hoc Dunnett test to determine significant differences. Significance was set at  $p < 0.05$ . For these statistical tests the n number used was the sample number, not the number of replicates. GraphPad Prism 4 software (GraphPad Software Inc, San Diego, USA) was used to perform the statistical analysis.

## **Chapter 3**

### ***Development of culture response methods***

# **Development of culture response methods**

## **3.1 Determination of J-HCET cell line population doubling time**

### **3.1.1 Introduction**

Measurement of the population doubling time is used to quantify the response of the cells to different inhibitory or stimulatory factors, such as variations in nutrient concentration or hormonal effects in medium or toxic drug exposure (Freshney, 1994). Studies were conducted to determine the effects that an animal product free human keratinocyte growth supplement (HKGS-V2), has upon the doubling time of the J-HCET corneal cell line. Unlike HKGS supplements which contain animal derived products, HKGS-V2 supplements require the use of a collagen matrix to aid initial cellular attachment upon seeding. Cascade Biologics Ltd produce an attachment matrix (type I collagen; TIC) specifically for use with the HKGS-V2 supplements, however, the use of an alternative fibronectin-collagen (FNC) matrix produced by AthenaES was investigated as a potential substitute. The population doubling times were, therefore, determined for J-HCET cultured with HKGS and HKGS-V2 supplemented media on both coated and uncoated flasks.

### **3.1.2 Materials and Methods**

Human corneal epithelial cells (J-HCET) cultured in either HKGS or HKGS-V2 supplemented growth media (section 2.1.7.1) were seeded into 25cm<sup>2</sup> flasks

(uncoated, TIC or FNC pre-coated as recommended by the manufacturer) at a density of  $4 \times 10^4$  cells per flask in 5ml of medium. Triplicate flasks were established for each time point. Cultures were maintained at  $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$  (v/v) in air and refed with fresh medium every 48 hours. During the experiment J-HCET were harvested after 24 hours of growth and subsequently every 24 hours up to 120 hours. The total cell number was determined using a haemocytometer (section 2.2.3.2). The number of population doublings in 24 hours was calculated using the following equation.

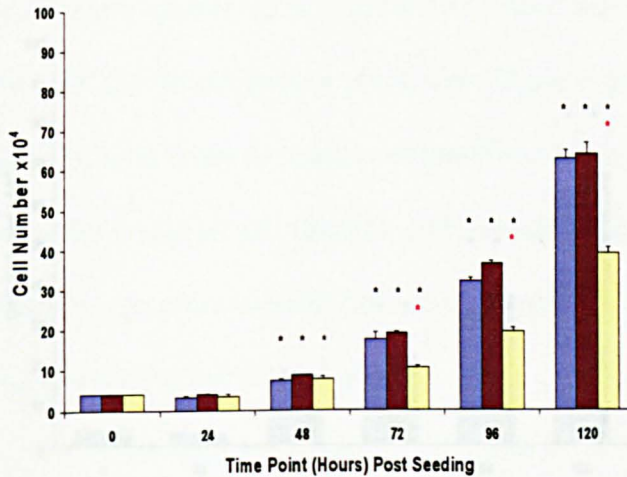
$$\frac{24}{\left( \sqrt[3]{\frac{\text{Total cell number at 96 hours}}{\text{Total cell number at 24 hours}}} \right) - 1}$$

### 3.1.3 Results

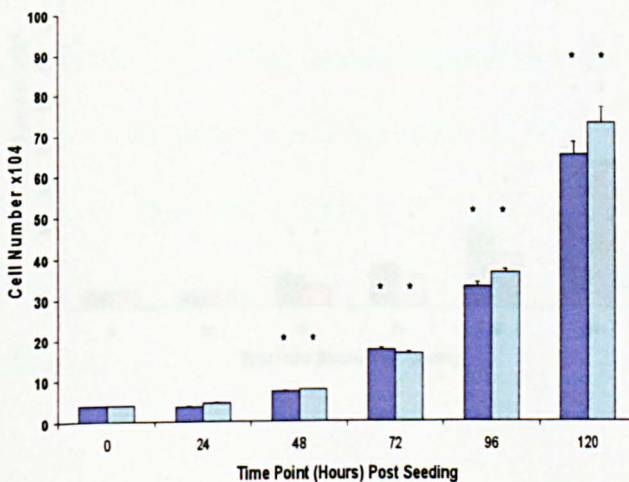
At 24 hours post seeding the cell number in the flasks had not significantly changed from the initial number seeded (figure 3.01), indicating a possible lack of attachment of J-HCET cells and/or a low initial rate of proliferation (i.e. an initial lag recovery phase post seeding) as a result of trypsinisation. In the subsequent 24 hours, (48 hrs post seeding) the cell number of both HKGS and HKGS-V2 cultures had significantly increased with a population doubling time of  $22 \pm 0.03$  and  $21 \pm 0.5$  hours for HKGS or HKGS-V2 supplements respectively (figure 3.01). If the recommended TIC matrix was not applied to the culture flasks when, HKGS-V2 supplements were employed, the population doubling time was determined as  $31 \pm 1.8$  hours. The addition of a TIC matrix to flasks, when using the HKGS supplements, did not



significantly alter the population doubling time (figure 3.02) from that of the HKGS cultures grown on uncoated flasks.



**Figure 3.01 :** Growth curves of J-HCET cultured in Epilife media containing either HKGS or HKGS-V2 supplements with or without a collagen matrix over 120 hours. The J-HCET cell number increased significantly (\* =  $P < 0.05$ ) with respect to 0 hour cultures when grown in either HKGS or HKGS-V2 supplemented media. There was no significant difference in cell number at each time point between the two supplements. From 72 to 120 hours the number of cells present in HKGS-V2 supplemented medium without a collagen coating was significantly (\* =  $P < 0.05$ ) lower compared to HKGS-V2 supplemented medium with a collagen coating (mean+S.D, n=4).

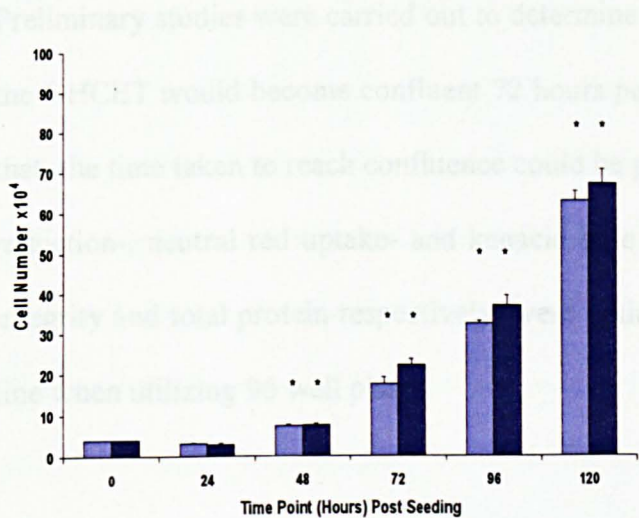


**Figure 3.02** Growth curves of J-HCET cultured in HKGS supplemented growth media in standard culture flasks or with a collagen coating over 120 hours. The J-HCET cell population increased significantly (\* =  $P < 0.05$ ) with respect to 0 hour cultures when cultured with or without matrix coating. There was no significant difference between the cell numbers at each time point between the two supplements. (mean+S.D, n=4).

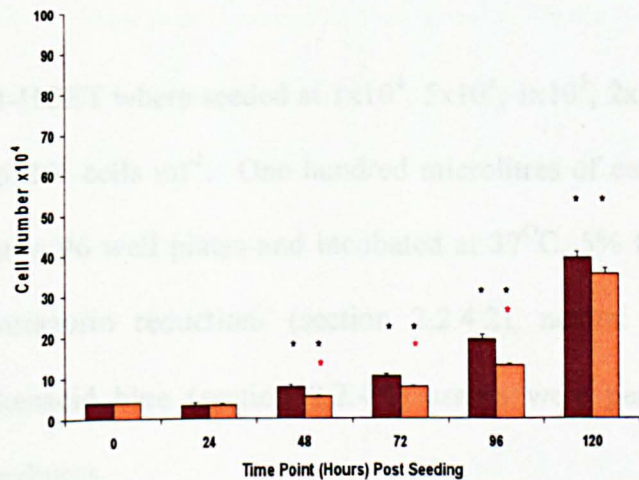
When an FNC matrix was employed the cell number had not significantly changed over the initial 24 hour period from the initial number seeded (figure 3.03 & 3.04), indicating a either low attachment or a low rate of proliferation, i.e. an initial lag recovery phase post seeding in this first 24 hours. The population doubling time for



J-HCET cultured in HKGS or HKGS-V2 supplemented media in FNC pre-coated flasks was determined as  $18\pm0.8$  and  $43\pm1.3$  hours respectively (figure 3.03 & 3.04), i.e. the FNC was not a substitute for the designated coating.



**Figure 3.03** Growth curves of J-HCET cultured in HKGS supplemented growth media in standard culture flasks ■ or with a FNC coating ■ over 120 hours. The J-HCET cell population increased significantly (\* =  $P<0.05$ ) with respect to 0 hour cultures when cultured with or without matrix coating. No significant difference is observed between cell number at any time point in either FNC or standard flasks. (mean+S.D, n=4).



**Figure 3.04 :** Growth curves of J-HCET cultured in HKGS-V2 supplemented growth media in standard culture flasks ■ or with a FNC coating ■ over 120 hours. The J-HCET cell population increased significantly (\* =  $P<0.05$ ) with respect to 0 hour cultures when cultured with or without matrix coating. At 48 and subsequently up to 96 hours the number of cells present when the matrix was employed was significantly (\* =  $P<0.05$ ) lower compared to HKGS-V2 supplemented medium alone (mean+S.D, n=4).

## **3.2 J-HCET seeding density required for monolayer formation**

### **3.2.1 Introduction**

Preliminary studies were carried out to determine a suitable seeding density whereby the J-HCET would become confluent 72 hours post seeding. This was performed so that the time taken to reach confluence could be predicted. In addition, the resazurin reduction-, neutral red uptake- and kenacid blue assays for cell activity, membrane integrity and total protein respectively, were optimised for use with the J-HCET cell line when utilizing 96 well plates.

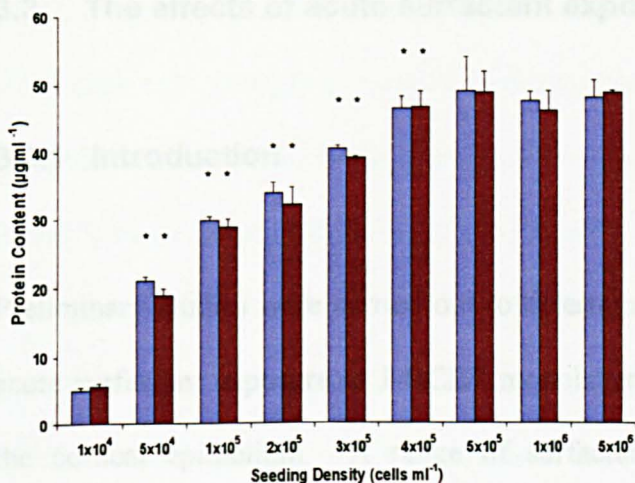
### **3.2.2 Materials and Methods**

J-HCET were seeded at  $1 \times 10^4$ ,  $5 \times 10^4$ ,  $1 \times 10^5$ ,  $2 \times 10^5$ ,  $3 \times 10^5$ ,  $4 \times 10^5$ ,  $5 \times 10^5$ ,  $1 \times 10^6$  and  $5 \times 10^6$  cells  $\text{ml}^{-1}$ . One hundred microlitres of cell suspension were seeded per well into 96 well plates and incubated at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$  (v/v) in air for 72 hours. The resazurin reduction- (section 2.2.4.2), neutral red uptake (section 2.2.4.5) and kenacid blue (section 2.2.4.1) assays were performed sequentially on the same cultures.

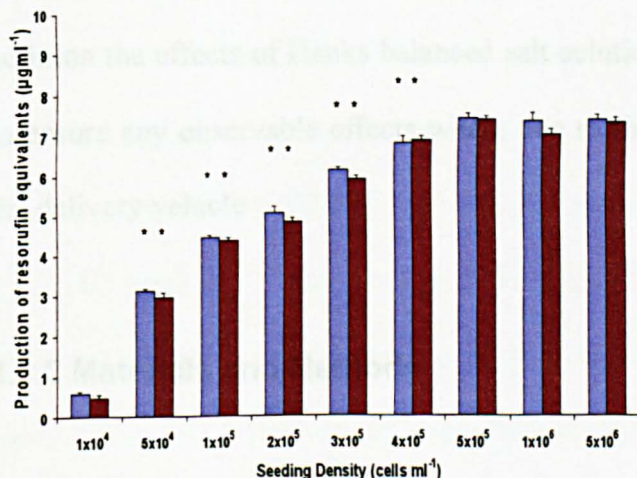
### **3.2.3 Results**

In addition to protein measurements, microscopic observations revealed that a seeding density of  $3 \times 10^5$  cells  $\text{ml}^{-1}$  resulted in a confluent J-HCET monolayer 72 hours post seeding and a protein content of  $40 \mu\text{gml}^{-1}$  (figure 3.05). At seeding

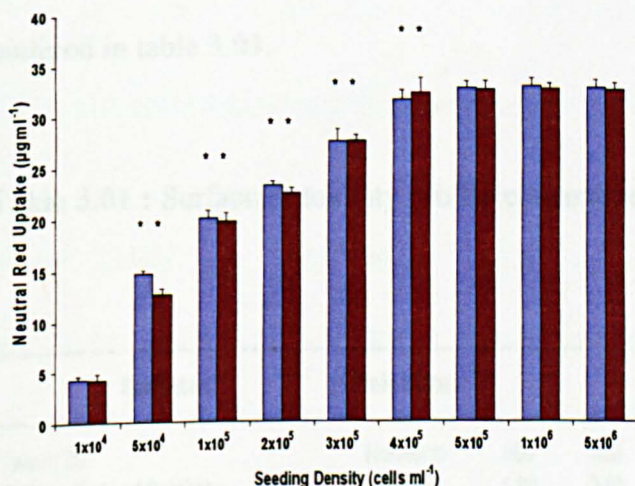
density above  $3 \times 10^5$  cells  $\text{ml}^{-1}$  ( $4 \times 10^5$ ,  $5 \times 10^5$ ,  $1 \times 10^6$  and  $5 \times 10^6$  cells  $\text{ml}^{-1}$ ) a monolayer was also established 72 hours post seeding with protein measurements reaching a plateau of approximately  $45 \mu\text{gml}^{-1}$ . This apparent inconsistency is associated with the increased cellular packing, within the J-HCET monolayer, that occurs at higher seeding densities. At seeding densities between  $1 \times 10^4$  and  $5 \times 10^4$  cells  $\text{ml}^{-1}$  resorufin production (resazurin reduction) increased proportionally with seeding density (figure 3.06). A plateau of resorufin production was achieved at seeding densities between  $5 \times 10^5$  and  $5 \times 10^6$  cells  $\text{ml}^{-1}$  which was consistent with the total well protein content reaching a maximum at these higher seeding densities (figure 3.05). At seeding densities between  $1 \times 10^4$  and  $4 \times 10^4$  cells  $\text{ml}^{-1}$  neutral red uptake increased proportionally with seeding density (figure 3.07). A plateau of neutral red uptake was achieved at seeding densities between  $4 \times 10^5$  and  $5 \times 10^6$  cells  $\text{ml}^{-1}$  which was consistent with protein content reaching a maximum. The profiles generated using the differing techniques are comparable. The levels of resorufin production, neutral red uptake and protein within the monolayers demonstrated no significant difference in J-HCET cultured in growth media supplemented with either HKGS or HKGS-V2.



**Figure 3.05 :** Effect of seeding density on protein content ( $\mu\text{gml}^{-1}$ ) of J-HCET cultures 72 hours post seeding grown in either HKGS ■ or HKGS-V2 ■ supplemented media. The protein content increased significantly (\* =  $P < 0.05$ ) with respect to previous seeding densities when cultured using either supplement type up to and including  $4 \times 10^5 \text{ cells ml}^{-1}$ . (mean+S.D, n=4).



**Figure 3.06 :** Effect of seeding density on resorufin production ( $\mu\text{gml}^{-1}$ ) of J-HCET cultures 72 hours post seeding grown in either HKGS ■ or HKGS-V2 ■ supplemented media. The resorufin production increased significantly (\* =  $P < 0.05$ ) with respect to previous seeding densities when cultured using either supplement type up to and including  $4 \times 10^5 \text{ cell ml}^{-1}$ . (mean+S.D, n=4).



**Figure 3.07 :** Effect of seeding density on neutral red uptake ( $\mu\text{gml}^{-1}$ ) of J-HCET cultures 72 hours post seeding grown in either HKGS ■ or HKGS-V2 ■ supplemented media. Neutral Red uptake increased significantly (\* =  $P < 0.05$ ) with respect to previous seeding densities when cultured using either supplement type up to and including  $4 \times 10^5 \text{ cell ml}^{-1}$ . (mean+S.D, n=3).

### 3.3 The effects of acute surfactant exposure on J-HCET monolayers

#### 3.3.1 Introduction

Preliminary studies were carried out to determine the amount of damage caused by acute surfactant exposure to J-HCET monolayers, used to model the basal layer of the corneal epithelium. A range of surfactant (SDS, T20, CAPB, and BAK) concentrations were employed, to determine a toxicity profile. This was assessed using the resazurin reduction, neutral red uptake and kenacid blue assays. In addition the effects of Hanks balanced salt solution (HBSS) exposure was examined to ensure any observable effects were due to the surfactant concentrations and not the delivery vehicle.

#### 3.3.2 Materials and Methods

Immediately prior to use, test surfactants (SDS, T20, CAPB or BAK) were suspended in HBSS (containing 1mM calcium chloride) to create concentrations as outlined in table 3.01.

**Table 3.01 : Surfactant toxicity profile concentration ranges.**

Surfactant	Ionic Status	Concentration (mgml <sup>-1</sup> ) in HBSS						
Tween 20	Non-ionic	500	450	400	350	300	250	200
Sodium Dodecyl Sulphate	Anionic	1.60	0.80	0.40	0.20	0.10	0.05	0.025
Cocamidopropylbentaine	Amphoteric	0.50	0.45	0.40	0.35	0.30	0.25	0.2
Benzalkonium Chloride	Cationic	0.02	0.0175	0.015	0.0125	0.01	0.0075	0.005



J-HCET were seeded into the central 54 wells of five 96 well plates were seeded at  $3 \times 10^5$  cells  $\text{ml}^{-1}$  (100 $\mu\text{l}$ ) for each surfactant. Phosphate buffered saline (100 $\mu\text{l}$ ) was applied to the outer 36 wells to prevent excessive evaporation of growth medium. Plates were incubated for 72 hours at 37°C and 5%  $\text{CO}_2$  (v/v) in air to allow the cells to attach and attain confluency. For each surfactant one plate was assayed following the 72 hour growth period, providing a pre-exposure control for that chemical. Medium was aspirated and the resazurin reduction (section 2.2.4.2), neutral red (section 2.2.4.5) and kenacid blue (section 2.2.4.1) assays performed. The medium was aspirated from the remaining plates and 200 $\mu\text{l}$  of each of the surfactant concentrations or HBSS (table 3.01) applied for 60 seconds. Wells were aspirated and the monolayers washed twice with 200 $\mu\text{l}$  of HBSS (60 seconds) containing 1mM calcium chloride. HBSS was aspirated and 200 $\mu\text{l}$  of cell culture media (pre-warmed to 37°C) applied. Plates were incubated at 37°C and 5%  $\text{CO}_2$  (v/v) in air. The resazurin reduction, neutral red uptake and kenacid blue assays were performed every 24 hours on different replicate plates up to 96 hours.

### **3.3.3 Results**

#### **3.3.3.1 Hanks balanced salt solution (HBSS) delivery vehicle**

HBSS (containing 1mM calcium chloride) exposure was not associated with significant alterations in resorufin production, neutral red uptake or protein content of J-HCET monolayers cultured in media containing either HKGS or HKGS-V2 supplements (table 3.02).

**Table 3.02 :** Effects of HBSS exposure on cell activity (Res), membrane integrity (NRU) and total protein content of J-HCET monolayers cultured in media containing either HKGS or HKGS-V2 supplements (mean±S.D., n=4).

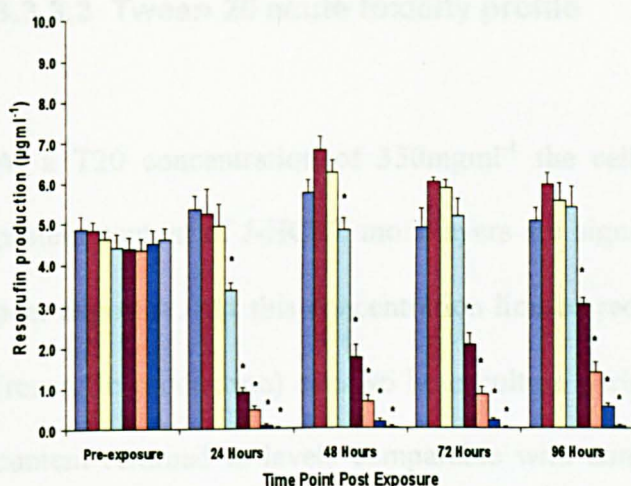
Supplement	Exposure	Parameter	Time point post exposure				
			Pre-exposure	24 Hours	48 Hours	72 Hours	96 Hours
HKGS	Medium	Resorufin ( $\mu\text{gm}^{-1}$ )	5±0.3	5±0.3	5±0.3	5±0.5	5±0.3
		Neutral Red ( $\mu\text{gm}^{-1}$ )	21±1.5	22±1.0	22±0.8	23±0.3	23±0.5
		Protein ( $\mu\text{gm}^{-1}$ )	31±3.7	32±2	33±2.4	33±1.4	32±0.6
	HBSS	Resorufin ( $\mu\text{gm}^{-1}$ )	5±0.3	5±0.5	5±0.2	5±0.3	5±0.3
		Neutral Red ( $\mu\text{gm}^{-1}$ )	21±0.9	23±0.9	22±0.4	22±0.7	23±1.3
		Protein ( $\mu\text{gm}^{-1}$ )	32±3.5	33±0.9	33±2.3	33±1.8	32±1.8
HKGS-V2	Medium	Resorufin ( $\mu\text{gm}^{-1}$ )	5±0.2	5±0.6	5±0.6	5±0.2	5±0.1
		Neutral Red ( $\mu\text{gm}^{-1}$ )	21±1.9	21±2.6	20±2.3	22±1.1	21±0.5
		Protein ( $\mu\text{gm}^{-1}$ )	33±4.1	34±3.4	32±2.9	33±2.5	31±2.6
	HBSS	Resorufin ( $\mu\text{gm}^{-1}$ )	5±0.4	5±1.2	5±0.8	5±0.9	5±0.5
		Neutral Red ( $\mu\text{gm}^{-1}$ )	21±0.6	22±2.3	23±0.8	21±0.5	22±0.9
		Protein ( $\mu\text{gm}^{-1}$ )	33±1.2	32±3.6	34±2.5	33±1.2	30±2.5

### 3.3.3.2 Sodium dodecyl sulphate acute toxicity profile

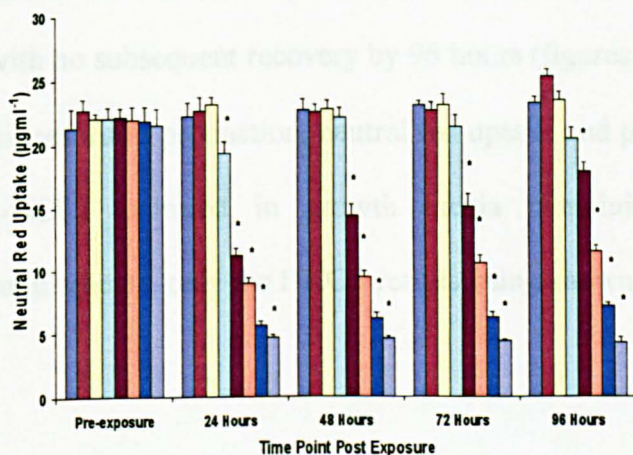
SDS at a concentration of 0.1mgml<sup>-1</sup> caused the cell activity, membrane integrity and protein content of J-HCET monolayers to be significantly (P<0.05) reduced, 24 hours post exposure, with subsequent recovery by 72 hours post exposure to levels comparable with control cultures (figure 3.08). At SDS concentrations of 0.2, 0.4, and 0.8mgml<sup>-1</sup> resorufin production, neutral red uptake and protein content is significantly (P<0.05) reduced 24 hours post exposure and did not recover fully by 96 hours (figures 3.08 to 3.10). At these concentrations recovery (in proportion to original exposure concentration) is observed but all measured parameters (resorufin, neutral red and protein) remain significantly (P<0.05) lower than controls. At a SDS concentration of 1.6mgml<sup>-1</sup> all measured parameters were significantly reduced (P<0.05) 24 hours post exposure with no subsequent recovery over 96 hours. The

toxicity profiles for resorufin production, neutral red uptake and protein content were comparable for J-HCET cultured in growth medium containing either HKGS or HKGS-V2 supplements, (figures 3.08 to 3.10) only the HKGS being shown as for the seeding density study (figures 3.05 to 3.07).

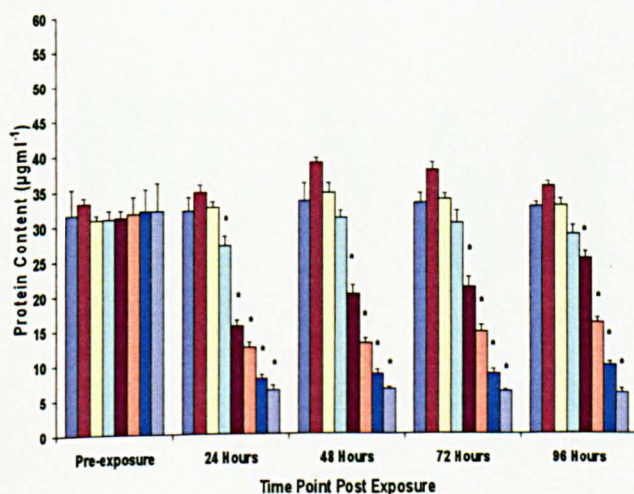




**Figure 3.08** : Production of resorufin ( $\mu\text{gml}^{-1}$ ) of J-HCET monolayers cultured in medium containing HKGS supplements over 96 hours subsequent to acute (60 seconds) sodium dodecyl sulphate exposure ■ Control, ■  $0.025\text{mgml}^{-1}$ , ■  $0.05\text{mgml}^{-1}$ , ■  $0.1\text{mgml}^{-1}$ , ■  $0.2\text{mgml}^{-1}$ , ■  $0.4\text{mgml}^{-1}$ , ■  $0.8\text{mgml}^{-1}$ , and ■  $1.6\text{mgml}^{-1}$  where \* denotes a significant ( $P<0.05$ ) decrease in resorufin production compared to control cultures (mean+S.D.,  $n=4$ ).



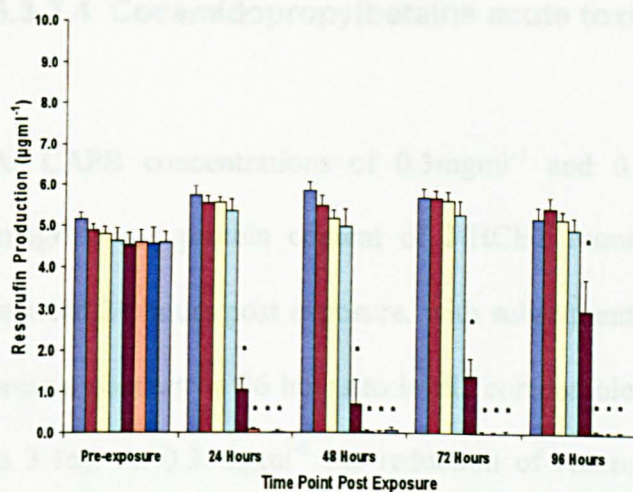
**Figure 3.09** : Neutral red uptake ( $\mu\text{gml}^{-1}$ ) of J-HCET monolayers cultured in medium containing HKGS supplements over 96 hours subsequent to acute (60 seconds) sodium dodecyl sulphate exposure ■ Control, ■  $0.025\text{mgml}^{-1}$ , ■  $0.05\text{mgml}^{-1}$ , ■  $0.1\text{mgml}^{-1}$ , ■  $0.2\text{mgml}^{-1}$ , ■  $0.4\text{mgml}^{-1}$ , ■  $0.8\text{mgml}^{-1}$ , and ■  $1.6\text{mgml}^{-1}$  where \* denotes a significant ( $P<0.05$ ) decrease in neutral red uptake compared to control cultures (mean+S.D.,  $n=3$ ).



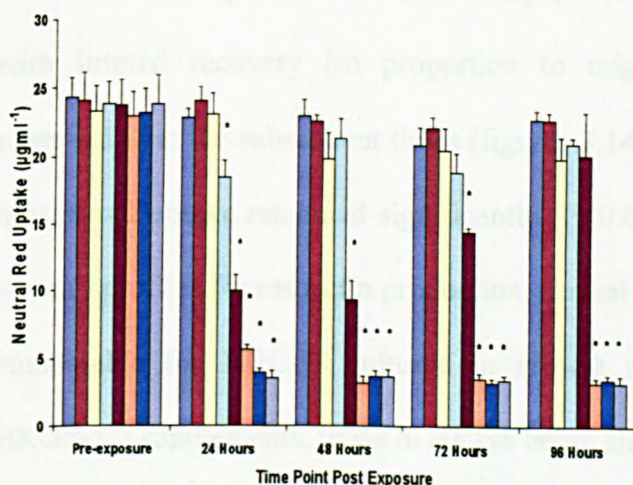
**Figure 3.10** : Protein content ( $\mu\text{gml}^{-1}$ ) of J-HCET monolayers cultured in medium containing HKGS supplements over 96 hours subsequent to acute (60 seconds) sodium dodecyl sulphate exposure ■ Control, ■  $0.025\text{mgml}^{-1}$ , ■  $0.05\text{mgml}^{-1}$ , ■  $0.1\text{mgml}^{-1}$ , ■  $0.2\text{mgml}^{-1}$ , ■  $0.4\text{mgml}^{-1}$ , ■  $0.8\text{mgml}^{-1}$ , and ■  $1.6\text{mgml}^{-1}$  where \* denotes a significant ( $P<0.05$ ) decrease in protein content compared to control cultures (mean+S.D.,  $n=4$ ).

### **3.3.3.3 Tween 20 acute toxicity profile**

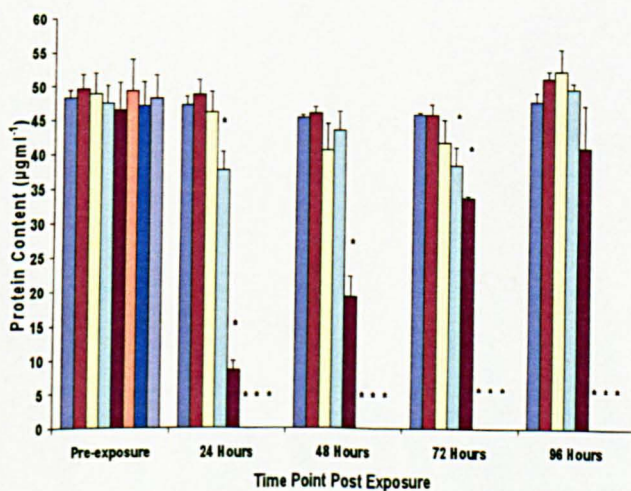
At a T20 concentration of  $350\text{mgml}^{-1}$  the cell activity, membrane integrity and protein content of J-HCET monolayers are significantly ( $P<0.05$ ) reduced 24 hours post exposure. At this concentration limited recovery was observed in cell activity (resorufin production) over 96 hour culture period. Membrane integrity and protein content returned to levels comparable with control cultures by 96 hours. At T20 concentrations of 400, 450 and  $500\text{mgml}^{-1}$  all measured parameters (resorufin, neutral red and protein) were significantly reduced ( $P<0.05$ ) 24 hours post exposure with no subsequent recovery by 96 hours (figures 3.11 to 3.13). The toxicity profiles for resorufin production, neutral red uptake and protein content were comparable for J-HCET cultured in growth media containing either HKGS or HKGS-V2 supplements, only the HKGS results being shown.



**Figure 3.11** : Production of resorufin equivalents ( $\mu\text{gml}^{-1}$ ) of J-HCET monolayers cultured in medium containing HKGS supplements over 96 hours subsequent to acute (60 seconds) tween 20 exposure ■ Control, ■  $200\text{mgml}^{-1}$ , ■  $250\text{mgml}^{-1}$ , ■  $300\text{mgml}^{-1}$ , ■  $350\text{mgml}^{-1}$ , ■  $400\text{mgml}^{-1}$ , ■  $450\text{mgml}^{-1}$ , and ■  $500\text{mgml}^{-1}$  where \* denotes a significant ( $P<0.05$ ) decrease in resorufin production to control cultures (mean+S.D.,  $n=4$ ).



**Figure 3.12** : Neutral red uptake ( $\mu\text{gml}^{-1}$ ) of J-HCET monolayers cultured in medium containing HKGS supplements over 96 hours subsequent to acute (60 seconds) tween 20 exposure ■ Control, ■  $200\text{mgml}^{-1}$ , ■  $250\text{mgml}^{-1}$ , ■  $300\text{mgml}^{-1}$ , ■  $350\text{mgml}^{-1}$ , ■  $400\text{mgml}^{-1}$ , ■  $450\text{mgml}^{-1}$ , and ■  $500\text{mgml}^{-1}$  where \* denotes a significant ( $P<0.05$ ) decrease in neutral red uptake compared to control cultures (mean+S.D.,  $n=3$ ).

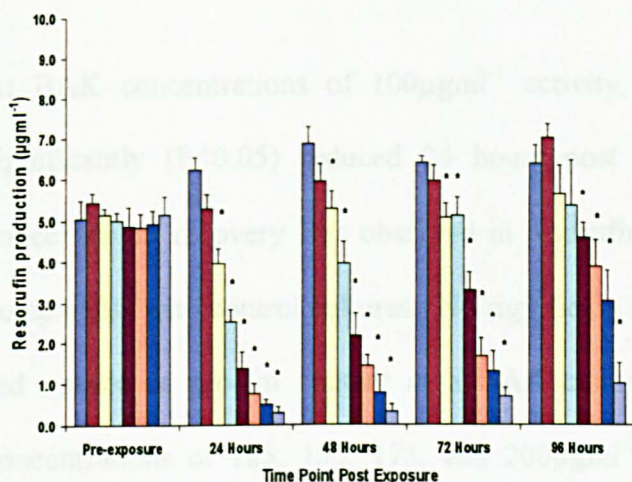


**Figure 3.13** : Protein content ( $\mu\text{gml}^{-1}$ ) of J-HCET monolayers cultured in medium containing HKGS supplements over 96 hours subsequent to acute (60 seconds) tween 20 exposure ■ Control, ■  $200\text{mgml}^{-1}$ , ■  $250\text{mgml}^{-1}$ , ■  $300\text{mgml}^{-1}$ , ■  $350\text{mgml}^{-1}$ , ■  $400\text{mgml}^{-1}$ , ■  $450\text{mgml}^{-1}$ , and ■  $500\text{mgml}^{-1}$  where \* denotes a significant ( $P<0.05$ ) decrease in protein content compared to control cultures (mean+S.D.,  $n=4$ ).

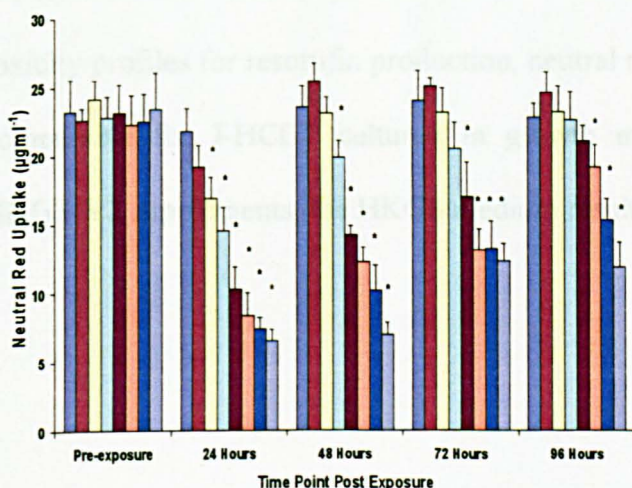


#### **3.3.3.4 Cocamidopropylbetaine acute toxicity profile**

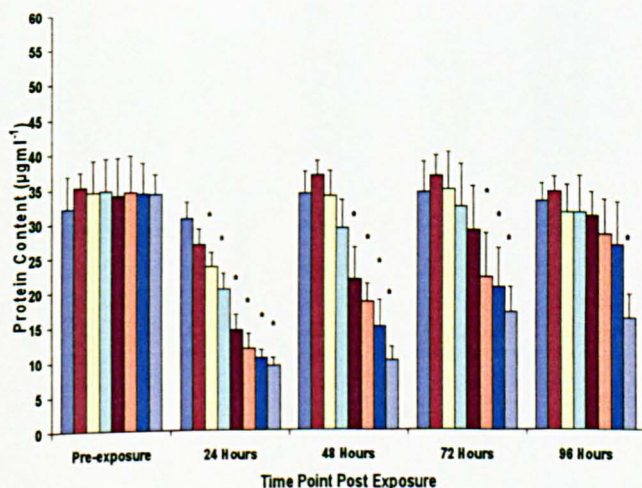
At CAPB concentrations of  $0.3\text{mgml}^{-1}$  and  $0.35\text{mgml}^{-1}$  the activity, membrane integrity and protein content of J-HCET monolayers was significantly ( $P<0.05$ ) reduced 24 hours post exposure, with subsequent recovery in neutral red uptake and protein content by 96 hours to levels comparable with control cultures (figures 3.14 to 3.16). At  $0.35\text{mgml}^{-1}$  the reduction of resorufin production did not recover by 96hrs. At CAPB concentrations of 0.40, 0.45 and  $0.50\text{mgml}^{-1}$  resorufin production and neutral red uptake were significantly ( $P<0.05$ ) reduced 24 hours post exposure, with limited recovery (in proportion to original exposure concentration) was observed over the subsequent times (figures 3.14 to 3.16). Resorufin production and neutral red uptake remained significantly ( $P<0.05$ ) lower than control cultures. The toxicity profiles for resorufin production, neutral red uptake and protein content were comparable for J-HCET cultured in growth media containing either HKGS or HKGS-V2 supplements, those in HKGS being shown.



**Figure 3.14** : Production of resorufin ( $\mu\text{gml}^{-1}$ ) of J-HCET monolayers cultured in medium containing HKGS supplements over 96 hours subsequent to acute (60 seconds) cocamidopropylbetaine exposure. Legend: Control,  $0.20\text{mgml}^{-1}$ ,  $0.25\text{mgml}^{-1}$ ,  $0.30\text{mgml}^{-1}$ ,  $0.35\text{mgml}^{-1}$ ,  $0.40\text{mgml}^{-1}$ ,  $0.45\text{mgml}^{-1}$ , and  $0.50\text{mgml}^{-1}$  where \* denotes a significant ( $P<0.05$ ) decrease in resorufin production to control cultures (mean+S.D.,  $n=4$ ).



**Figure 3.15** : Neutral red uptake ( $\mu\text{gml}^{-1}$ ) of J-HCET monolayers cultured in medium containing HKGS supplements over 96 hours subsequent to acute (60 seconds) cocamidopropylbetaine exposure. Legend: Control,  $0.20\text{mgml}^{-1}$ ,  $0.25\text{mgml}^{-1}$ ,  $0.30\text{mgml}^{-1}$ ,  $0.35\text{mgml}^{-1}$ ,  $0.40\text{mgml}^{-1}$ ,  $0.45\text{mgml}^{-1}$ , and  $0.50\text{mgml}^{-1}$  where \* denotes a significant ( $P<0.05$ ) decrease in neutral red uptake compared to control cultures (mean+S.D.,  $n=3$ ).

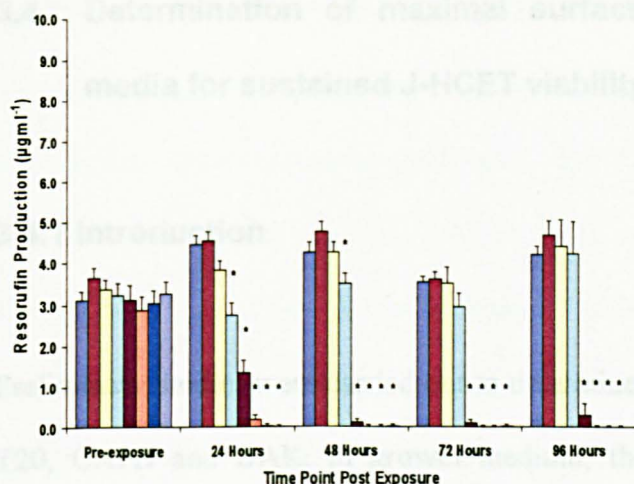


**Figure 3.16** : Protein content ( $\mu\text{gml}^{-1}$ ) of J-HCET monolayers cultured in medium containing HKGS supplements over 96 hours subsequent to acute (60 seconds) cocamidopropylbetaine exposure. Legend: Control,  $0.20\text{mgml}^{-1}$ ,  $0.25\text{mgml}^{-1}$ ,  $0.30\text{mgml}^{-1}$ ,  $0.35\text{mgml}^{-1}$ ,  $0.40\text{mgml}^{-1}$ ,  $0.45\text{mgml}^{-1}$ , and  $0.50\text{mgml}^{-1}$  where \* denotes a significant ( $P<0.05$ ) decrease in protein content compared to control cultures (mean+S.D.,  $n=4$ ).

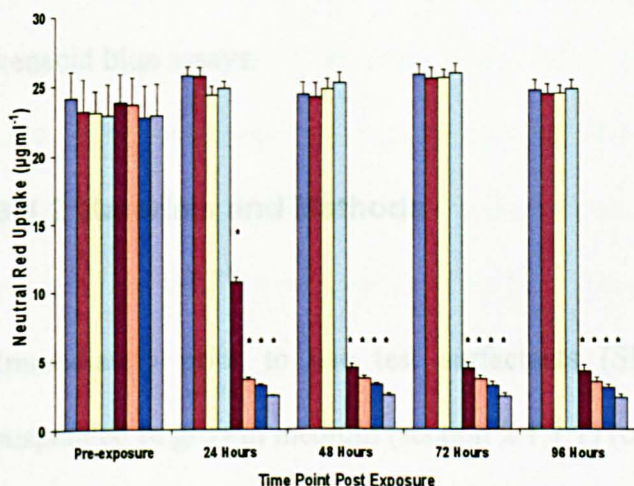
### **3.3.3.5 Benzalkonium chloride acute toxicity profile**

At BAK concentrations of  $100\mu\text{gml}^{-1}$  activity, of the J-HCET monolayers was significantly ( $P<0.05$ ) reduced 24 hours post exposure (figure 3.17). At this concentration recovery was observed in resorufin production by 72 hours to levels comparable with control cultures. No significant alterations were observed in neutral red uptake or protein content at a BAK concentration of  $100\mu\text{gml}^{-1}$ . At BAK concentrations of 125, 150, 175, and  $200\mu\text{gml}^{-1}$  resorufin production, neutral red uptake and protein content were significantly reduced ( $P<0.05$ ) 24 hours post exposure with no subsequent recover by 96 hours (figure 3.17, 3.18 and 3.19). The toxicity profiles for resorufin production, neutral red uptake and protein content were comparable for J-HCET cultured in growth media containing either HKGS or HKGS-V2 supplements, the HKGS medium results being shown.

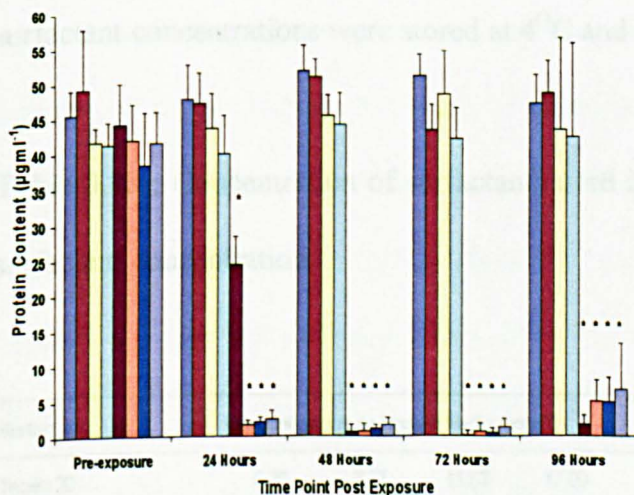




**Figure 3.17** : Production of resorufin equivalents ( $\mu\text{gml}^{-1}$ ) of J-HCET monolayers cultured in medium containing HKGS supplements over 96 hours subsequent to acute (60 seconds) benzalkonium chloride exposure. Control,  $50\mu\text{gml}^{-1}$ ,  $75\mu\text{gml}^{-1}$ ,  $100\mu\text{gml}^{-1}$ ,  $125\mu\text{gml}^{-1}$ ,  $150\mu\text{gml}^{-1}$ ,  $175\mu\text{gml}^{-1}$ , and  $200\mu\text{gml}^{-1}$  where \* denotes a significant ( $P<0.05$ ) decrease in resorufin production compared to control cultures (mean+S.D.,  $n=4$ ).



**Figure 3.18** : Neutral red uptake ( $\mu\text{gml}^{-1}$ ) of J-HCET monolayers cultured in medium containing HKGS supplements over 96 hours subsequent to acute (60 seconds) benzalkonium chloride exposure. Control,  $50\mu\text{gml}^{-1}$ ,  $75\mu\text{gml}^{-1}$ ,  $100\mu\text{gml}^{-1}$ ,  $125\mu\text{gml}^{-1}$ ,  $150\mu\text{gml}^{-1}$ ,  $175\mu\text{gml}^{-1}$ , and  $200\mu\text{gml}^{-1}$  where \* denotes a significant ( $P<0.05$ ) decrease in neutral red uptake compared to control cultures (mean+S.D.,  $n=3$ ).



**Figure 3.19** : Protein content ( $\mu\text{gml}^{-1}$ ) of J-HCET monolayers cultured in medium containing HKGS supplements over 96 hours subsequent to acute (60 seconds) benzalkonium chloride exposure. Control,  $50\mu\text{gml}^{-1}$ ,  $75\mu\text{gml}^{-1}$ ,  $100\mu\text{gml}^{-1}$ ,  $125\mu\text{gml}^{-1}$ ,  $150\mu\text{gml}^{-1}$ ,  $175\mu\text{gml}^{-1}$ , and  $200\mu\text{gml}^{-1}$  where \* denotes a significant ( $P<0.05$ ) decrease in protein content compared to control cultures (mean+S.D.,  $n=4$ ).

**3.4 Determination of maximal surfactant concentration in growth media for sustained J-HCET viability over 21 days.**

**3.4.1 Introduction**

Preliminary studies were carried out to determine the maximal concentration of SDS, T20, CAPB and BAK, in growth medium, that would allow sustained J-HCET viability over a 21 day exposure period. A range of concentrations were employed, to determine a dose response of toxicity, assessed using neutral red uptake and kenacid blue assays.

**3.4.2 Materials and Methods**

Immediately prior to use test surfactants (SDS, T20, CAPB or BAK), were suspended in growth medium (section 2.1.7.1) (containing 1mM calcium chloride) to give the concentrations as outlined in table 3.03. Surfactant concentrations were made up in medium containing either HKGS or HKGS-V2 supplements. Chronic surfactant concentrations were stored at 4°C and used within 4 weeks of preparation.

**Table 3.03 : Concentration of surfactants used in the determination of subcytotoxic surfactant concentration.**

Surfactant	Concentrations in Growth Media (µgml <sup>-1</sup> )								
Tween 20	5.35	7.87	11.57	17.00	25.00	36.75	54.02	79.41	116.73
Sodium Dodecyl Sulphate	1.85	2.72	3.99	5.86	8.62	12.67	18.63	27.39	40.26
Cocamidopropylbetaine	0.64	0.94	1.38	2.04	2.99	4.41	6.48	9.52	14.00
Benzalkonium Chloride	0.0025	0.0031	0.0046	0.0065	0.0044	0.0064	0.0139	0.0205	0.0301



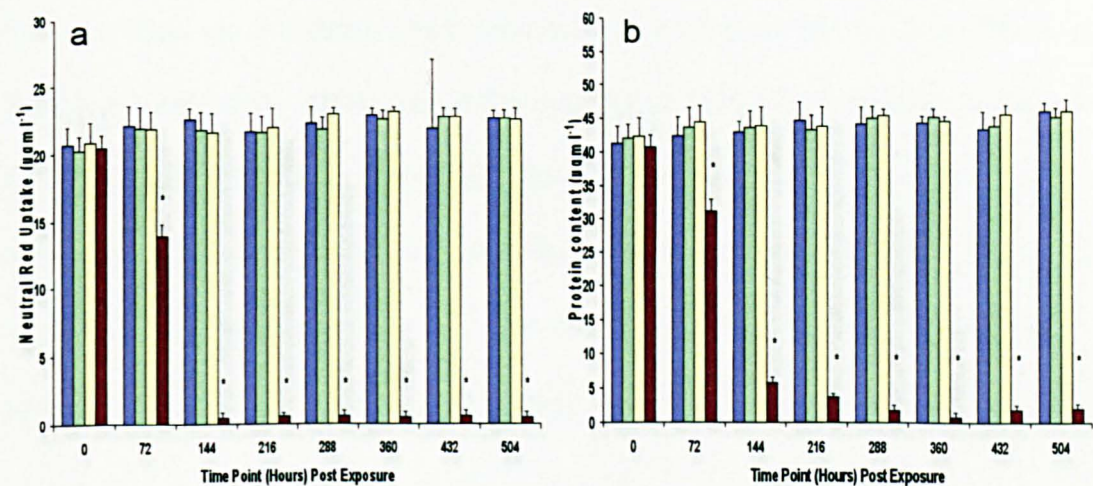
J-HCET were seeded into the central 54 wells of eight 96 well plates at  $3 \times 10^5$  cells  $\text{ml}^{-1}$  (100 $\mu\text{l}$ ). Phosphate buffered saline (PBS) (100 $\mu\text{l}$ ) was applied to the outer 36 wells to prevent excessive evaporation of growth media. Plates were incubated for 72 hours at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$  (v/v) to allow cells to attach and attain confluency. For each surfactant one plate was assayed, following the initial 72 hour growth to confluence period, providing a pre-exposure control for that chemical. Medium was aspirated and the neutral red (section 2.2.4.5) and kenacid blue (section 2.2.4.1) assays performed. The medium in the remaining plates was replaced with 200 $\mu\text{l}$  of medium containing surfactant concentrations as outlined in table 3.03. For each concentration 6 wells were employed. Plates were refed with fresh culture media, containing the appropriate surfactant, every 72 hours and incubated at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$  (v/v) in air. The neutral red uptake and kenacid blue assays were performed on one plate every 72 hours over 21 days (504 hours).

### 3.4.3 Results

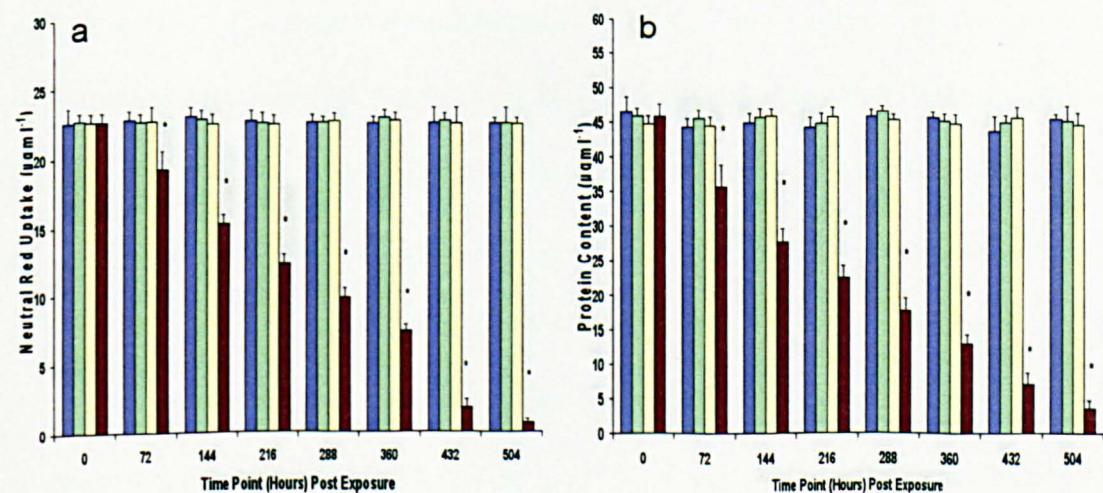
SDS (figure 3.20a/b) and T20 (figure 3.21a/b) concentrations employed above  $4\mu\text{gml}^{-1}$  and  $25\mu\text{gml}^{-1}$  respectively, resulted in significantly reduced neutral red uptake and protein content following 72 hours exposure, continuing up to 504 hours. Below these concentrations no significant effects on neutral red uptake or protein content were observed. CAPB (figure 3.22a/b) concentrations employed above  $2.99\mu\text{gml}^{-1}$  resulted in a significant ( $P < 0.05$ ) reduction in neutral red uptake and protein content, compared to the control, after 288 hours up to 504 hours. Concentrations below  $2.99\mu\text{gml}^{-1}$  had no significant effect on neutral red uptake or protein content. BAK (figure 3.23a/b) concentrations used above  $0.0025\mu\text{gml}^{-1}$

significantly ( $P < 0.05$ ) reduced neutral red uptake and protein content after 144 hours up to 504 hours. Concentrations below  $0.0025 \mu\text{gml}^{-1}$  had no significant effect on neutral red uptake or protein content. The toxicity profiles for neutral red uptake and protein content were comparable for J-HCET cultured in growth media containing either HKGS or HKGS-V2 supplements, the HKGS medium results being shown.

**Figure 3.20a/b :** Neutral red uptake ( $\mu\text{gml}^{-1}$ ) (a) and protein content ( $\mu\text{gml}^{-1}$ ) (b) of J-HCET monolayers cultured in HKGS supplemented media containing  $\blacksquare$   $0\mu\text{gml}^{-1}$  (control)  $\blacksquare$   $2.72\mu\text{gml}^{-1}$   $\blacksquare$   $3.99\mu\text{gml}^{-1}$  or  $\blacksquare$   $5.86\mu\text{gml}^{-1}$  sodium dodecyl sulphate over 504 hours where \* denotes a significant ( $P<0.05$ ) decrease in neutral red uptake or protein content compared to control cultures (mean+S.D.,  $n=3$ ).

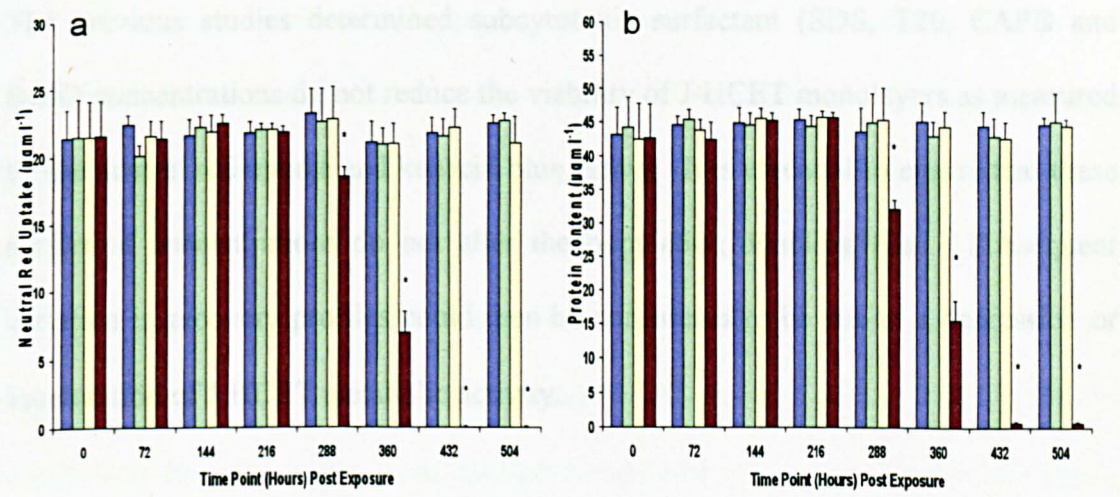


**Figure 3.21a/b :** Neutral red uptake ( $\mu\text{gml}^{-1}$ ) (a) and protein content ( $\mu\text{gml}^{-1}$ ) (b) of J-HCET monolayers cultured in HKGS supplemented media containing  $\blacksquare$   $0\mu\text{gml}^{-1}$  (control)  $\blacksquare$   $17\mu\text{gml}^{-1}$   $\blacksquare$   $25\mu\text{gml}^{-1}$  or  $\blacksquare$   $36.75\mu\text{gml}^{-1}$  tween 20 over 504 hours where \* denotes a significant ( $P<0.05$ ) decrease in neutral red uptake or protein content compared to control cultures (mean+S.D.,  $n=3$ ).

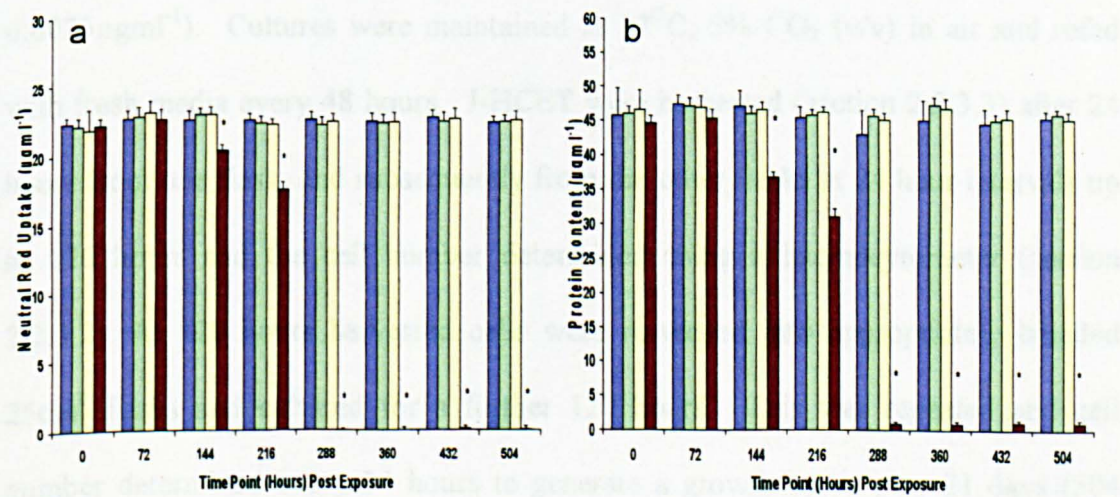




**Figure 3.22a/b :** Neutral red uptake ( $\mu\text{gml}^{-1}$ ) (a) and protein content ( $\mu\text{gml}^{-1}$ ) (b) of J-HCET monolayers cultured in HKGS supplemented media containing  $\blacksquare$   $0\mu\text{gml}^{-1}$  (control)  $\blacksquare$   $2.04\mu\text{gml}^{-1}$   $\blacksquare$   $2.99\mu\text{gml}^{-1}$  or  $\blacksquare$   $4.41\mu\text{gml}^{-1}$  cocamidopropylbetaine over 504 hours where \* denotes a significant ( $P<0.05$ ) decrease in neutral red uptake or protein content compared to control cultures (mean+S.D.,  $n=3$ ).



**Figure 3.23a/b :** Neutral red uptake ( $\mu\text{gml}^{-1}$ ) (a) and protein content ( $\mu\text{gml}^{-1}$ ) (b) of J-HCET monolayers cultured in HKGS supplemented media containing  $\blacksquare$   $0\mu\text{gml}^{-1}$  (control)  $\blacksquare$   $0.0025\mu\text{gml}^{-1}$   $\blacksquare$   $0.0031\mu\text{gml}^{-1}$  or  $\blacksquare$   $0.0046\mu\text{gml}^{-1}$  benzalkonium chloride over 504 hours where \* denotes a significant ( $P<0.05$ ) decrease in neutral red uptake or protein content compared to control cultures (mean+S.D.,  $n=3$ ).



### **3.5 J-HCET doubling time when cultured in growth media containing subcytotoxic surfactant concentrations**

#### **3.5.1 Introduction**

The previous studies determined subcytotoxic surfactant (SDS, T20, CAPB and BAK) concentrations do not reduce the viability of J-HCET monolayers as measured by the neutral red uptake and kenacid blue assays. It is essential to ensure that these surfactant concentrations do not alter the population doubling time. Subsequent variation in recovery profiles could then be considered to be due to a depression or acceleration of J-HCET metabolic activity.

#### **3.5.2 Materials and Methods**

Chronic exposure medium was prepared. Human corneal epithelial cells (J-HCET) were seeded into 25cm<sup>2</sup> flasks (uncoated or TIC pre-coated) at a density of 4x10<sup>4</sup> cells per flask in 5ml of medium. Triplicate flasks were established for each time point and surfactant type (SDS; 4µgml<sup>-1</sup>, T20; 25µgml<sup>-1</sup>, CAPB; 3µgml<sup>-1</sup>, BAK; 0.0025µgml<sup>-1</sup>). Cultures were maintained at 37°C, 5% CO<sub>2</sub> (v/v) in air and refed with fresh media every 48 hours. J-HCET were harvested (section 2.2.3.3) after 24 hours from one flask, and subsequently from the other flasks at 24 hour intervals up to 120 hours and the cell number determined using a haemocytometer (section 2.2.3.2). At 120 hours harvested cells were re-seeded into appropriately labelled 25cm<sup>2</sup> flasks and cultured for a further 120 hours. This was repeated and cell number determined every 24 hours to generate a growth curve over 21 days (504

hours). The population doubling time was calculated using the previous equation (section 3.1).

### **3.5.3 Results**

J-HCET cells were exposed to subcytotoxic concentrations of SDS, T20, CAPB and BAK for a 21 day period. All surfactant exposed J-HCET population doubling times were compared to their corresponding control cultures. For cells grown in medium containing HKGS supplements, surfactant exposure did not alter the population doubling times (table 3.04). For cells cultured in medium containing HKGS-V2 supplements on a TIC matrix coating, surfactant exposure significantly increased the population doubling times from that of the control (no exposure) cultures (table 3.04). For cells cultured in medium containing HKGS-V2 supplements alone, surfactant exposure did not alter the population doubling times (table 3.04). This suggests that the subcytotoxic surfactant concentrations have degraded the matrix pre-coated to the tissue culture plasticware hindering cellular adhesion.

**Table 3.04** : Population doubling times of J-HCET cultured in subcytotoxic concentrations of surfactants over 21 days (mean±S.D, n=4).

Supplement	Surfactant	Concentration ( $\mu\text{gml}^{-1}$ )	Doubling Time (Hours) Between Days			
			0 - 5	5 - 10	10 - 15	15 - 21
HKGS	None (Control)	N/A	22±1.3	21±0.5	21±0.1	21±0.1
	Sodium Dodecyl Sulphate	4.00	22±0.9	21±0.3	20±0.3	21±0.1
	Tween 20	25.00	22±1.3	21±0.1	21±0.3	21±0.3
	Cocamidopropylbetaine	3.00	21±0.6	21±0.6	20±0.4	21±0.5
	Benzalkonium Chloride	0.0025	21±1.4	20±0.2	21±0.1	21±0.3
HKGS-V2 <sup>M</sup>	None (Control)	N/A	21±0.5	20±0.4	21±0.8	20±0.9
	Sodium Dodecyl Sulphate	4.00	26±0.2	25±0.4	24±0.5	25±0.2
	Tween 20	25.00	26±0.6	25±1.6	25±0.6	25±0.9
	Cocamidopropylbetaine	3.00	26±0.7	26±0.1	26±1.2	26±1.1
	Benzalkonium Chloride	0.0025	25±0.1	26±0.2	25±0.7	26±1.3
HKGS-V2	None (Control)	N/A	30±5.6	30±0.5	28±0.1	29±0.3
	Sodium Dodecyl Sulphate	4.00	31±0.8	29±0.5	27±1.4	28±1.1
	Tween 20	25.00	31±0.3	29±0.3	29±0.5	30±0.8
	Cocamidopropylbetaine	3.00	30±0.1	29±0.1	28±1.9	30±0.7
	Benzalkonium Chloride	0.0025	29±0.3	28±0.3	27±0.8	29±0.5

<sup>M</sup> Culture flasks were pre-coated with the recommended type I collagen

### **3.6 Evaluation of the effects of chronic subcytotoxic surfactant exposure to a human corneal cell line**

#### **3.6.1 Introduction**

This study aims to investigate the chronic adverse effects of exogenous chemicals. Initially the viability of the J-HCET monolayers was monitored following chronic exposure to subcytotoxic concentrations of four representative surfactants; SDS, T20, CAPB and BAK. Interactions between surfactants were investigated to determine if chronic exposure to one surfactant, at subcytotoxic concentrations, rendered the J-HCET monolayer more or less susceptible to a subsequent acute exposure to the same or different surfactant. Measurements of monolayer viability were made using the resazurin reduction, neutral red uptake and kenacid blue assays.

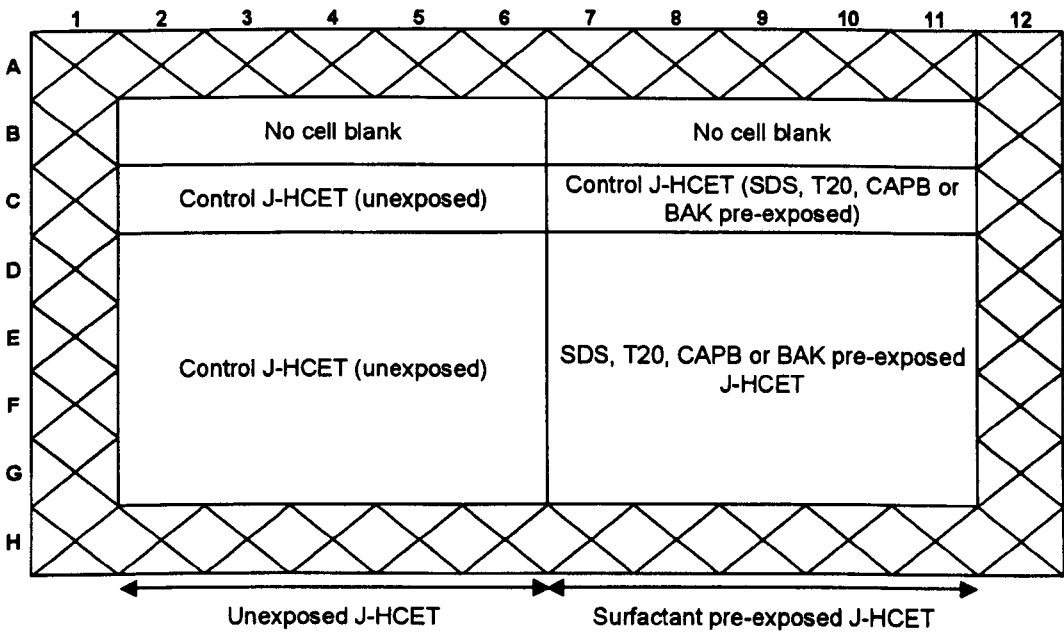
#### **3.6.2 Materials and Methods**

Culture flasks (80cm<sup>3</sup>) were seeded with  $5 \times 10^5$  of J-HCET cells in normal culture medium and incubated for 24 hours at 37°C, 5% (v/v) CO<sub>2</sub> in air. Medium or medium containing subcytotoxic surfactant concentrations was added to each flask where appropriate. Cultures were maintained at 37°C, 5% (v/v) CO<sub>2</sub> in air, re-fed with the appropriate medium every 72 hours over 432 hours (18 days) and subcultured once cells were 70-80% confluent. Cells were monitored on a daily basis at low magnification (x10 objective) to ascertain the level of cell growth, cell morphology and to check for contamination. Following the 18 day exposure, J-HCET cells were harvested from large culture flasks. The central 54 wells of five 96



well plates were seeded at  $3 \times 10^5$  cells  $\text{ml}^{-1}$  (100 $\mu\text{l}$ ) for each surfactant, as outlined in figure 3.24. Phosphate buffered saline (100 $\mu\text{l}$ ) was applied to the outer 36 wells to prevent excessive evaporation of growth medium. Plates were incubated for 72 hours at 37°C and 5% CO<sub>2</sub> (v/v) in air to allow the cells to attach and attain confluency. At this point the cells have been cultured in subcytotoxic concentrations of the respective surfactant for 21 days.

**Figure 3.24 :** J-HCET seeding template to assess the effects of chronic subcytotoxic surfactant exposure.



For each surfactant one plate underwent resazurin reduction assay then neutral red uptake and finally kenacid blue (total protein) assays. This provided a pre-exposure control for that chemical. In the remaining plates the medium was replaced in row C with either medium C2-6 or medium with chronic levels of the respective surfactant C7-11. Rows D to G (figure 3.24) were aspirated and 200 $\mu\text{l}$  of each surfactant, at a

predetermined concentration (section 3.3, SDS; 0.1mgml<sup>-1</sup>, T20; 325mgml<sup>-1</sup>, CAPB; 0.3mgml<sup>-1</sup> and BAK; 100µgml<sup>-1</sup>), was applied for 60 seconds. Cells were washed with 200µl of PBS (2 x 60 seconds). Medium or medium containing subcytotoxic surfactant concentrations (200µl) was applied. Plates were incubated at 37°C and 5% CO<sub>2</sub> (v/v) in air. The resazurin reduction, neutral red uptake and kenacid blue assays were performed on replicate plates at 24 hour intervals, up to 96 hours.

### **3.6.3 Results**

#### **3.6.3.1 Effects of chronic subcytotoxic surfactant exposure on J-HCET resorufin production, neutral red uptake and protein content.**

Control un-exposed and pre-exposed cultures (figure 3.24, row C) showed the same levels of resorufin production, neutral red uptake or protein content (table 3.05), over the course of the experiment. Resorufin, neutral red and protein levels were comparable in J-HCET cultured in growth media containing HKGS (table 3.05) or HKGS-V2 supplements (not shown). For the remaining experiments protein content data has not been shown.

**Table 3.05 :** Resorufin production, neutral red uptake and protein content of surfactant pre-exposed J-HCET monolayers cultured in medium containing HKGS supplements (mean±S.D, n=3).

Culture Conditions	Parameter	Time Point				
		Pre-exposure	24 Hours	48 Hours	72 Hours	96 Hours
Control (no exposure)	Resorufin (µgml <sup>-1</sup> )	3.7±0.2	4.2±0.2	4.7±0.3	4.9±0.3	4.8±0.3
	Neutral Red (µgml <sup>-1</sup> )	17±2.2	17±1.4	19±0.9	19±1.7	19±1.5
	Protein (µgml <sup>-1</sup> )	51±6.1	50±3.8	58±2.4	58±4.9	59±4.3
Sodium Dodecyl Sulphate (4µgml <sup>-1</sup> )	Resorufin (µgml <sup>-1</sup> )	3.6±0.1	4.3±0.2	4.8±0.1	4.9±0.1	4.9±0.1
	Neutral Red (µgml <sup>-1</sup> )	18±1.2	17±1.6	20±1.0	21±1.1	20±1.1
	Protein (µgml <sup>-1</sup> )	53.6±3.4	51.6±4.6	58.3±3.9	59.5±3.1	58.0±3.0
Tween 20 (25µgml <sup>-1</sup> )	Resorufin (µgml <sup>-1</sup> )	3.1±0.2	4.2±0.2	4.8±0.2	5.1±0.5	4.9±0.2
	Neutral Red (µgml <sup>-1</sup> )	17±1.0	17±0.3	20±1.1	21±1.9	22±1.5
	Protein (µgml <sup>-1</sup> )	51±2.9	50±0.9	56±3.0	59±5.3	63±4.3
Cocamidopropylbetaine (3µgml <sup>-1</sup> )	Resorufin (µgml <sup>-1</sup> )	3.8±0.2	4.2±0.1	4.4±0.3	4.7±0.2	4.8±0.2
	Neutral Red (µgml <sup>-1</sup> )	17±2.4	17±1.0	19±0.6	20±2.0	20±1.8
	Protein (µgml <sup>-1</sup> )	52±6.8	50±3.0	56±1.6	59±5.6	59±5.1
Benzalkonium Chloride (0.0025µgml <sup>-1</sup> )	Resorufin (µgml <sup>-1</sup> )	3.7±0.2	4.2±0.4	4.9±0.2	4.9±0.2	4.8±0.4
	Neutral Red (µgml <sup>-1</sup> )	17±1.5	17±1.6	19±0.9	21±1.4	20±1.8
	Protein (µgml <sup>-1</sup> )	49±4.3	50±4.4	56±2.5	60±3.9	59±5.0

### **3.6.3.2 Effects of SDS ( $0.4\mu\text{gml}^{-1}$ ) pre-exposure on surfactant toxicity profile**

Chronic subcytotoxic SDS exposure (figures 3.25 and 3.26) ( $4\mu\text{gml}^{-1}$ ) significantly ( $P<0.05$ ) increased the toxicity of subsequent acute exposures of SDS ( $0.1\text{mgml}^{-1}$ ), CAPB ( $0.3\text{mgml}^{-1}$ ) and BAK ( $100\mu\text{gml}^{-1}$ ) demonstrated by reductions in the production of resorufin, neutral red uptake and protein content compared to control cultures. Resorufin, neutral red and protein levels were comparable in J-HCET cultured in growth media containing HKGS or HKGS-V2 supplements.

### **3.6.3.3 Effects of T20 ( $25\mu\text{gml}^{-1}$ ) pre-exposure on surfactant toxicity profile**

Tween 20 pre-exposure (figure 3.25 and 3.26) significantly ( $P<0.05$ ) decreased the toxicity of subsequent acute exposures of SDS ( $0.1\text{mgml}^{-1}$ ), CAPB ( $0.3\text{mgml}^{-1}$ ) and BAK ( $100\mu\text{gml}^{-1}$ ). This was demonstrated by an increase in the production of resorufin, neutral red uptake and protein content at 24 and 48 hours post exposure for SDS and BAK, and up to 72 hours for CAPB, compared to control cultures. At 96 hours post exposure J-HCET monolayers exposed to acute T20 concentration demonstrate a significant ( $P<0.05$ ) decrease in resorufin production, neutral red uptake and total protein content compared to control cultures. Resorufin, neutral red and protein levels were comparable in J-HCET cultured in growth media containing HKGS or HKGS-V2 supplements.

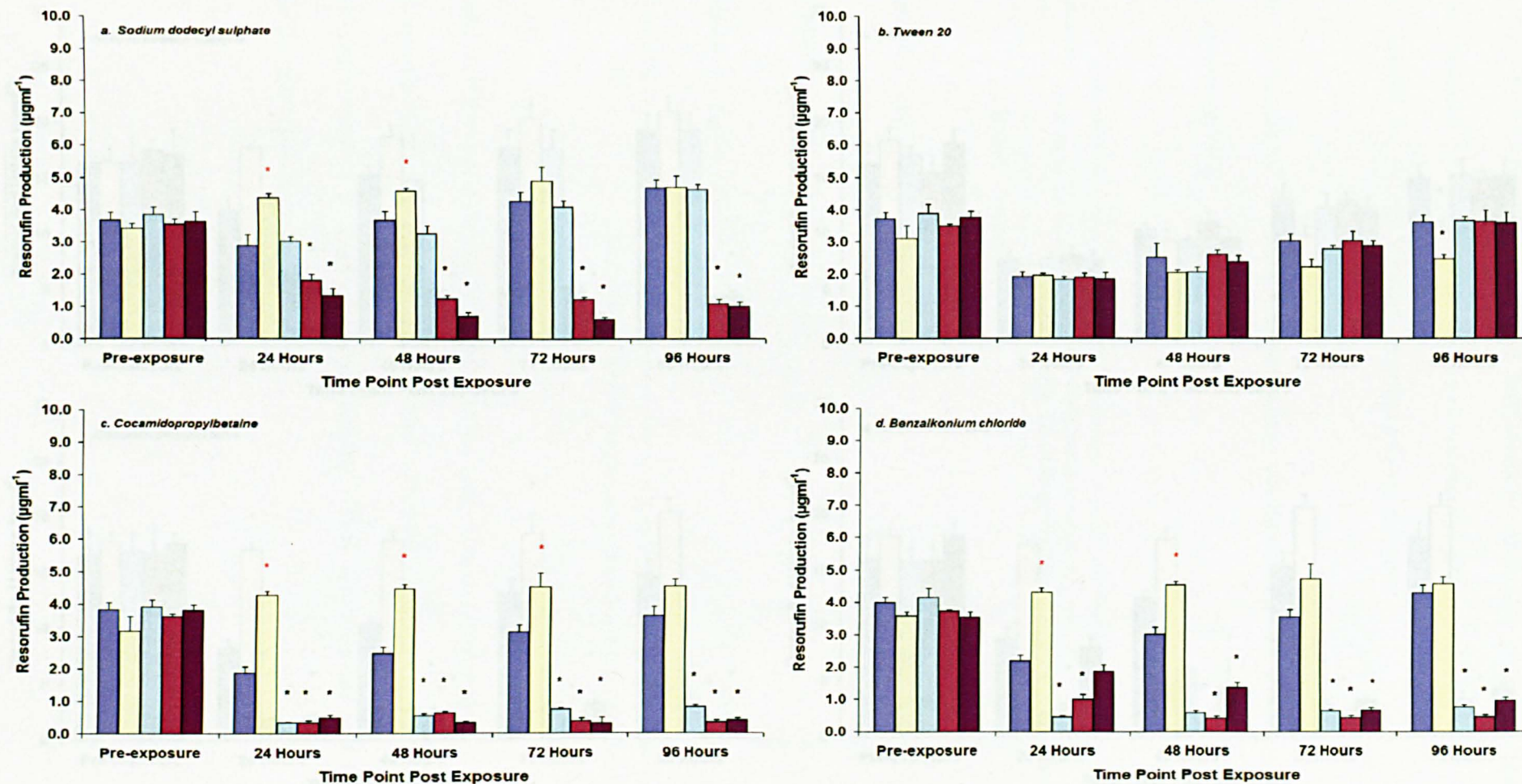
#### **3.6.3.4 Effects of CAPB ( $3\mu\text{gml}^{-1}$ ) pre-exposure on surfactant toxicity profile**

Subcytotoxic CAPB exposure (figure 3.25 and 3.26) significantly ( $P<0.05$ ) increased the toxicity of subsequent acute exposures of CAPB ( $0.3\text{mgml}^{-1}$ ) and BAK ( $100\mu\text{gml}^{-1}$ ) demonstrated by reductions in the production of resorufin, neutral red uptake and protein content up to 96 hours compared to control cultures. Pre-exposure to CAPB has no significant effects on resorufin production, neutral red uptake or protein content of J-HCET exposed to an acute concentrations or either SDS ( $0.1\text{mgml}^{-1}$ ) or T20 ( $325\text{mgml}^{-1}$ ). Resorufin, neutral red and protein levels were comparable in J-HCET cultured in growth media containing HKGS or HKGS-V2 supplements.

#### **3.6.3.5 Effects of BAK ( $0.0025\mu\text{gml}^{-1}$ ) pre-exposure on surfactant toxicity profile**

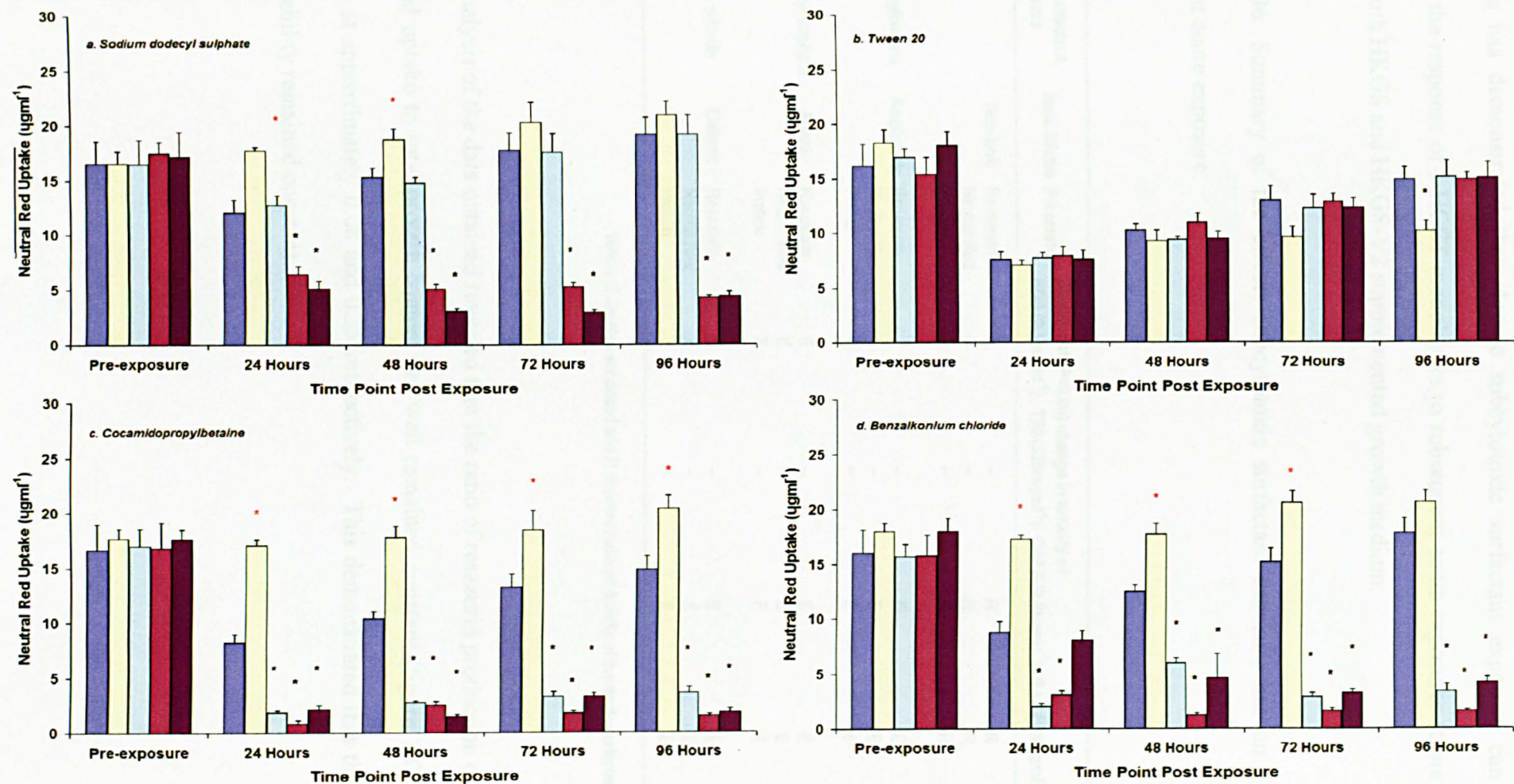
Chronic subcytotoxic BAK exposure (figure 3.25 and 3.26) significantly ( $P<0.05$ ) increased the toxicity of subsequent acute exposures of SDS ( $0.1\text{mgml}^{-1}$ ), CAPB ( $0.3\text{mgml}^{-1}$ ) and BAK ( $100\mu\text{gml}^{-1}$ ) demonstrated by reductions in the production of resorufin, neutral red uptake and protein content up to 96 hours compared to control cultures. Pre-exposure to BAK has no significant effects on resorufin production, neutral red uptake or protein content of J-HCET exposed to an acute concentrations of T20 ( $325\text{mgml}^{-1}$ ). Resorufin, neutral red and protein levels were comparable in J-HCET cultured in growth media containing HKGS or HKGS-V2 supplements.

**Figure 3.25 :** Resorufin production ( $\mu\text{gml}^{-1}$ ) of J-HCET monolayers cultured in media containing HKGS supplements exposed to subcytotoxic concentrations of surfactants ■ control culture, ■ sodium dodecyl sulphate, ■ tween 20, ■ cocamidopropylbetaine or, ■ benzalkonium chloride for 21 days and subsequently exposed (60 seconds) to acute concentrations of *a.*) Sodium dodecyl sulphate ( $0.1\text{mgml}^{-1}$ ), *b.*) Tween 20 ( $325\text{mgml}^{-1}$ ), *c.*) Cocamidopropylbetaine ( $0.3\text{mgml}^{-1}$ ) or *d.*) Benzalkonium chloride ( $100\mu\text{gml}^{-1}$ ) where \* denotes a significant ( $P<0.05$ ) decrease in resorufin and \* a significant ( $P<0.05$ ) increase in resorufin production compared to control cultures. ( mean+S.D (n=4)





**Figure 3.26 :** Neutral red uptake ( $\mu\text{gml}^{-1}$ ) of J-HCET monolayers cultured in media containing HKGS supplements exposed to subcytotoxic concentrations of surfactants ■ control culture, ■ sodium dodecyl sulphate, ■ tween 20, ■ cocamidopropylbetaine or, ■ benzalkonium chloride for 21 days and subsequently exposed (60 seconds) to acute concentrations of *a.*) Sodium dodecyl sulphate ( $0.1\text{mgml}^{-1}$ ), *b.*) Tween 20 ( $325\text{mgml}^{-1}$ ), *c.*) Cocamidopropylbetaine ( $0.3\text{mgml}^{-1}$ ) or *d.*) Benzalkonium chloride ( $100\mu\text{gml}^{-1}$ ) where \* denotes a significant ( $P<0.05$ ) decrease in resorufin and \* a significant ( $P<0.05$ ) increase in resorufin production compared to control cultures. ( mean+S.D (n=4)



This data has demonstrated that chronic subcytotoxic surfactant exposure can modulate the response of J-HCET monolayers to subsequent acute exposures (table 3.06) in both HKGS and HKGS-V2 supplemented growth medium.

**Table 3.06:** Summary of the effects subcytotoxic surfactant exposure has on a subsequent acute exposure.

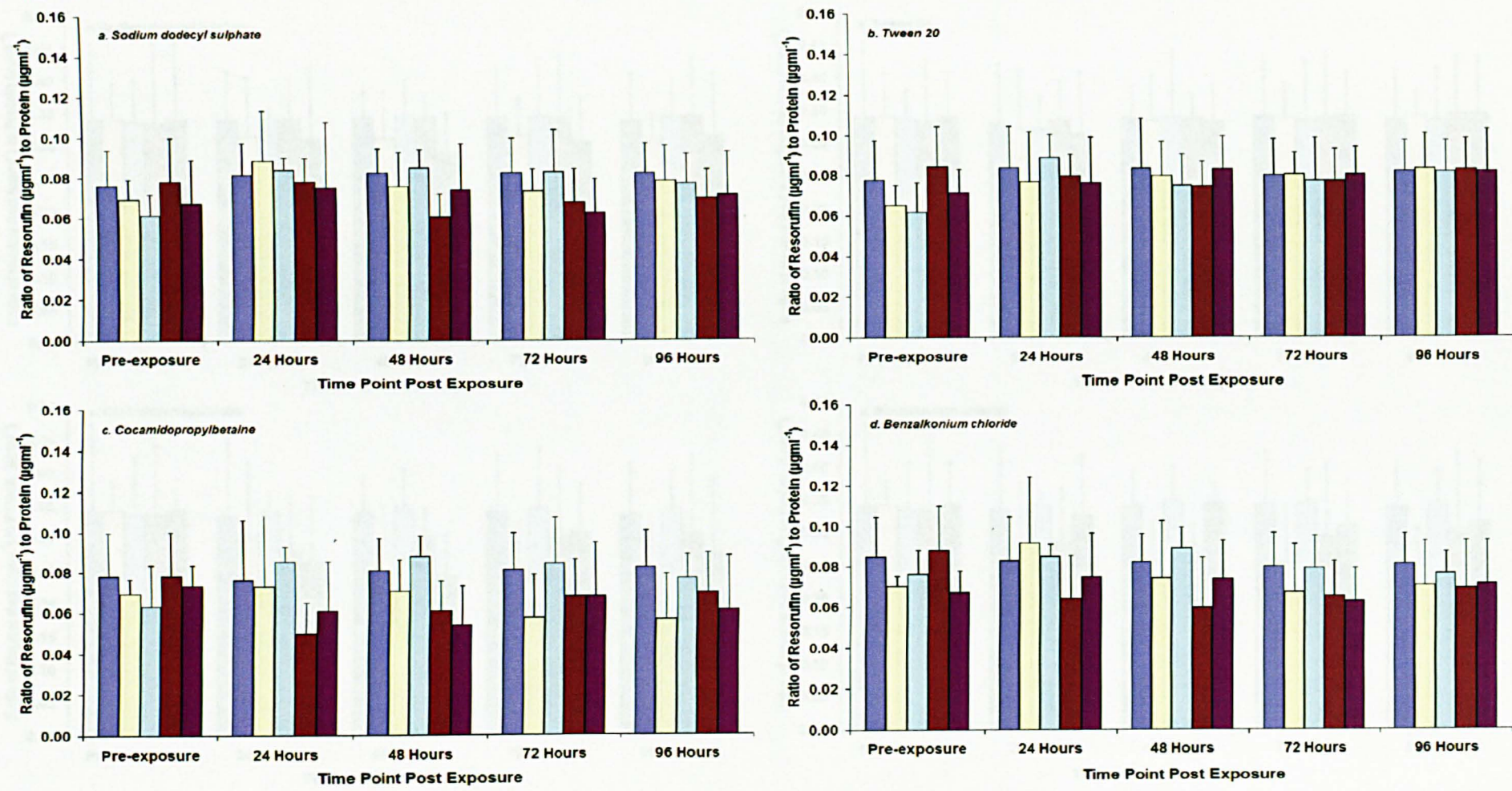
Chronic Surfactant Exposure	Ionic Status	Parameter	Significant (P<0.05) change in toxicity of			
			SDS (0.1mgml <sup>-1</sup> )	T20 (325mgml <sup>-1</sup> )	CAPB (0.3mgml <sup>-1</sup> )	BAK (100µgml <sup>-1</sup> )
Tween 20	Non-ionic	Resazurin	R	–	R	R
		Neutral Red	R	–	R	R
		Protein	R	–	R	R
Cocamidopropylbetaine	Amphoteric	Resazurin	–	–	E	E
		Neutral Red	–	–	E	E
		Protein	–	–	E	E
Sodium dodecyl sulphate	Anionic	Resazurin	E	–	E	E
		Neutral Red	E	–	E	E
		Protein	E	–	E	E
Benzalkonium chloride	Cationic	Resazurin	E	–	E	E
		Neutral Red	E	–	E	E
		Protein	E	–	E	E

Where E denotes enhanced and R denotes reduced toxicity of the acute surfactant.

Further analysis of the data obtained revealed that the ratio of resazurin production or neutral red uptake to total protein content per well remained constant (figure 3.07 and 3.08) at approximately 0.08 and 0.35 respectively. This demonstrated that the culture viability remained constant.

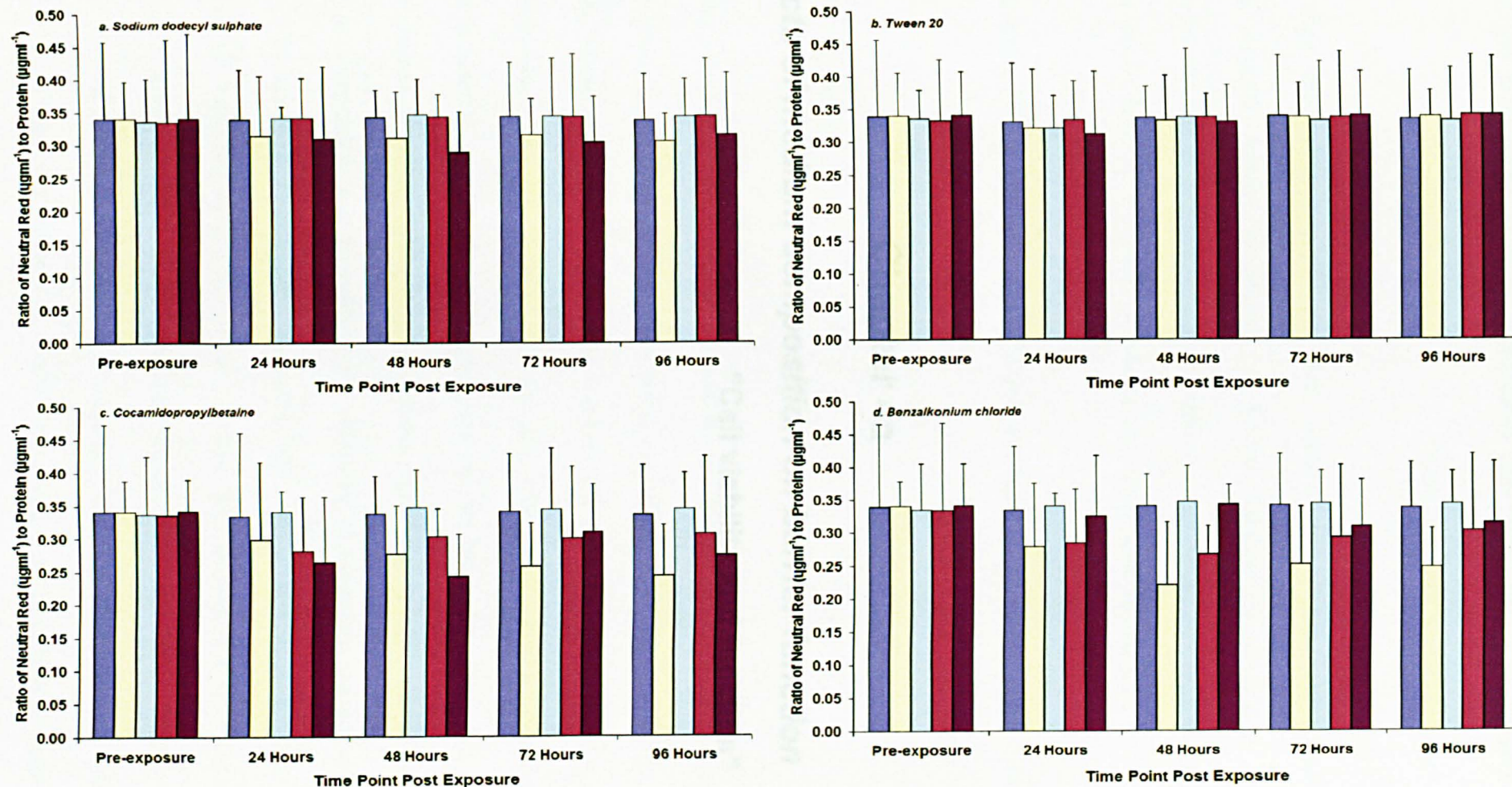


**Figure 3.27 :** Ratio of resorufin production ( $\mu\text{gml}^{-1}$ ) per microgram protein of J-HCET monolayers cultured in media containing HKGS supplements exposed to subcytotoxic concentrations of surfactants ■ control culture, ■ sodium dodecyl sulphate, ■ tween 20, ■ cocamidopropylbetaine or, ■ benzalkonium chloride for 21 days and subsequently exposed (60 seconds) to acute concentrations of *a.* Sodium dodecyl sulphate ( $0.1\text{mgml}^{-1}$ ), *b.* Tween 20 ( $325\text{mgml}^{-1}$ ), *c.* Cocamidopropylbetaine ( $0.3\text{mgml}^{-1}$ ) or *d.* Benzalkonium chloride ( $100\mu\text{gml}^{-1}$ ) (mean+S.D.,  $n=4$ ).





**Figure 3.28 :** Ratio of neutral red uptake ( $\mu\text{gml}^{-1}$ ) per microgram protein of J-HCET monolayers cultured in media containing HKGS supplements exposed to subcytotoxic concentrations of surfactants ■ control culture, ■ sodium dodecyl sulphate, ■ tween 20, ■ cocamidopropylbetaine or, ■ benzalkonium chloride for 21 days and subsequently exposed (60 seconds) to acute concentrations of *a.*) Sodium dodecyl sulphate ( $0.1\text{mgml}^{-1}$ ), *b.*) Tween 20 ( $325\text{mgml}^{-1}$ ), *c.*) Cocamidopropylbetaine ( $0.3\text{mgml}^{-1}$ ) or *d.*) Benzalkonium chloride ( $100\mu\text{gml}^{-1}$ ). (mean+S.D., n=4).



## **Chapter 4a**

***Effects of medium composition on barrier function***

**“Cell viability and function”**

# Effects of medium composition on barrier function

## “Cell viability and function”

The following chapter is divided into three sections, containing experiments concerning J-HCET activity in relation to barrier function, those related to morphological changes as a consequence of medium composition, and experiments relating the barrier function to adhesion molecule expression and localisation.

### 4.1 Optimal J-HCET seeding density for the formation of a competent barrier

#### 4.1.1 Introduction

Multicellular organisms contain various compositionally distinct fluid compartments, which are bounded by epithelial and endothelial cell sheets (Tsukita and Furuse, 1999). The corneal epithelium functions as a barrier that isolates the eye from the outside environment and pollutants in the tear film (Ban *et al.*, 2003). For the epithelium to function as a barrier, maintaining the distinct internal environment of each compartment, the paracellular pathway between adjacent cells in the sheet must be sealed in a regulated way to prevent the diffusion of unwanted solutes and solvents. The apical and basolateral domains must be differentiated to allow selective active transport across the sheet (Tsukita and Furuse, 1999). Tight junctions (TJ's; Zona adherens) are directly involved in paracellular barrier function regulation. The TJ's provide a continuous seal around the apical aspect of adjoining epithelial cells, thereby preventing the uninhibited passage of molecules between

adjacent epithelial cells via the paracellular pathway. When these junctions are compromised, as a result of chemical or physical injury, exogenously added sodium fluorescein (MW 361) can penetrate between the cells and into the layers beneath the apical epithelium and reveal, using ultra violet light, the location of damage in the human eye (Cotton and Zanvit, 1997; Zanvit *et al.*, 1999). In order to act as a model of the corneal epithelium it is essential that the J-HCET cell line is able to form an effective barrier to the passage of exogenous chemicals. Previous studies with this cell line have shown that the combined fluorescein leakage and resazurin assays can be effectively employed (Combes *et al.*, 1999; Moore *et al.*, 2005). Preliminary studies were carried out to determine the optimal J-HCET seeding density to allow the formation of a barrier to the passage of sodium fluorescein within 3 days *in vitro*. Studies were conducted to confirm the optimal J-HCET seeding density onto 0.45µm polycarbonate inserts (Nuncleon) to allow the formation of a barrier to the passage of sodium fluorescein, as previously employed by Moore *et al.*, (2005).

#### **4.1.2 Materials and Methods**

J-HCET (500µl) were seeded into Nunc Polycarbonate inserts at  $1 \times 10^4$ ,  $5 \times 10^4$ ,  $1 \times 10^5$ ,  $5 \times 10^5$ ,  $1 \times 10^6$  and  $5 \times 10^6$  cells  $\text{ml}^{-1}$ . Two inserts received 500µl of Epilife keratinocyte medium alone, i.e. were the no-cell insert controls. The eight inserts were placed into 24 well culture plates with 500µl of medium per well. Cultures were maintained at 37°C, 5% CO<sub>2</sub> (v/v) in air. After 72 hours in culture the leakage of fluorescein across the monolayer was determined using the combined fluorescein leakage- resazurin reduction assay (section 2.2.4.3). The leakage data is expressed as a percentage of the leakage across the no-cell insert set at 100% whilst the resazurin

reduction as equivalents of resorufin (section 2.2.4.2). The total cell protein content on the inserts was established using the kenacid blue assay (section 2.2.4.1).

### **4.1.3 Results**

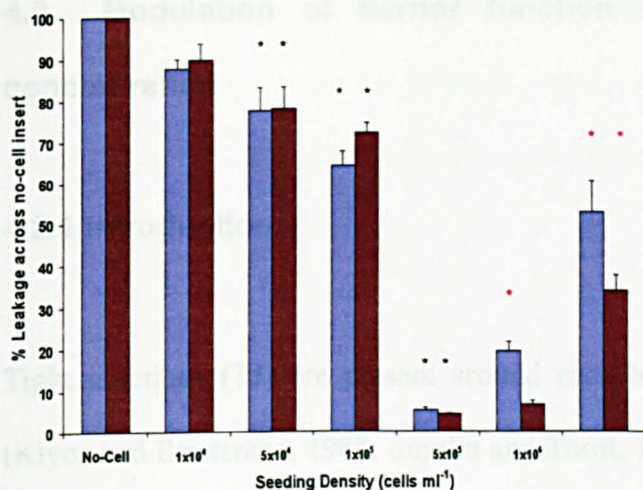
Permeability to sodium fluorescein decreased proportionally with increasing seeding density (figure 4.01). A visual inspection of the inserts could not confirm the presence of a totally confluent J-HCET monolayer, due to the opacity of the polycarbonate membrane. Seeding at  $1 \times 10^4$  cells  $\text{ml}^{-1}$  did not significantly alter the flux of fluorescein into the basolateral chamber. At seeding densities of  $5 \times 10^4$  to  $1 \times 10^5$  the leakage was significantly ( $P < 0.05$ ) reduced compared to the no cell control after 72 hours. The lowest flux occurred at  $5 \times 10^5$  cells  $\text{ml}^{-1}$  when either HKGS or HKGS-V2 supplemented medium was employed, giving  $5.2 \pm 0.53\%$  and  $4.2 \pm 0.11\%$  in HKGS and HKGS-V2 supplemented media respectively. This correlated with the resorufin production of approximately  $4 \mu\text{gml}^{-1}$  and total protein content of  $50 \mu\text{gml}^{-1}$  of the cultures, a plateau level that did not significantly increase with higher cell densities (figures 4.02 and 4.03). Seeding at a high density, whilst giving similar total cell protein and resazurin results, did adversely affect fluorescein leakage (figure 4.01).

### **4.1.4 Conclusions**

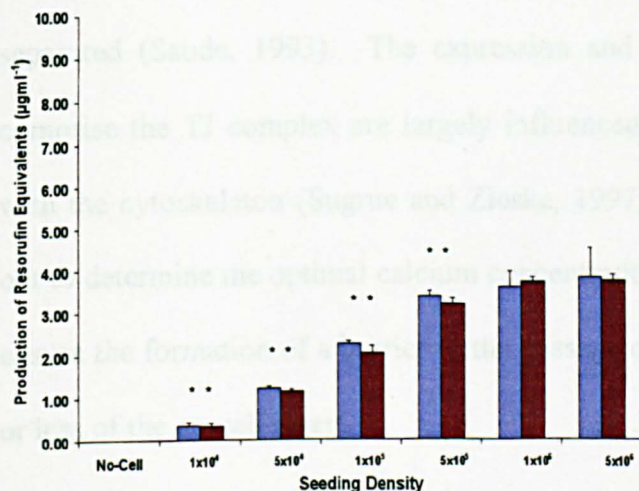
The formation of an effective barrier, *in vitro* depends upon formation of a confluent layer of cells. Studies with MDCK cells (Clothier and Samson 1996) have shown that if seeded at too high a density then adherence to parts of the culture surface was

compromised and that the small areas devoid of cells result in a high rate of fluorescein leakage. Therefore, the corneal cells act in the same way as previously observed with the MDCK cultures, namely that they need to “grow” to confluency to ensure a fully formed barrier *in vitro*.

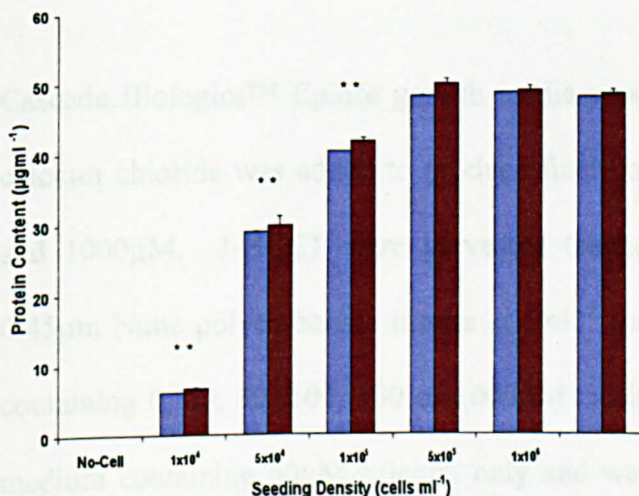




**Figure 4.01** : Effect of seeding density on fluorescein leakage across a J-HCET monolayer cultured in either HKGS ■ or HKGS-V2 ■. Monolayers formed from seeding densities over  $1 \times 10^4$  cells  $\text{ml}^{-1}$  significantly decreased the leakage of fluorescein (\* =  $P < 0.05$  compared to no-cell insert). Seeding at  $5 \times 10^5$  cells  $\text{ml}^{-1}$  gave the lowest flux of fluorescein. At higher seeding densities in HKGS media the leakage of fluorescein significantly increases proportionally (\* =  $P < 0.05$ ) to density. In HKGS-V2 supplemented media a significant increase in leakage (\* =  $P < 0.05$ ) is observed at a density of  $5 \times 10^6$  cells  $\text{ml}^{-1}$ . (mean  $\pm$  S.D,  $n=3$ ).



**Figure 4.02** : Effect of seeding density on resorufin production in the J-HCET monolayer cultured in either HKGS ■ or HKGS-V2 ■. Monolayers formed from seeding densities over  $1 \times 10^4$  cells  $\text{ml}^{-1}$  demonstrate a significant (\* =  $P < 0.05$ ) proportional increase in resorufin production up to a plateau level of  $4.0 \mu\text{gml}^{-1}$ . (mean  $\pm$  S.D,  $n=3$ ).



**Figure 4.03** : Effect of seeding density on total protein concentration in the J-HCET monolayer cultured in either HKGS ■ or HKGS-V2 ■ supplemented media. Monolayers formed from seeding densities over  $1 \times 10^4$  cells  $\text{ml}^{-1}$  demonstrate a significant (\* =  $P < 0.05$ ) proportional increase in protein content up to a plateau level of  $5 \times 10^5$  cells  $\text{ml}^{-1}$ . (mean  $\pm$  S.D,  $n=3$ ).



## **4.2 Modulation of barrier function by growth medium calcium concentration**

### **4.2.1 Introduction**

Tight junctions (TJ) are present around each human apical epithelial cell *in vivo* (Klyce and Beuerman, 1988; Smolin and Thoft, 1994). These prevent the movement of small molecules and ions between the tear film and the corneal stroma, which could otherwise result in corneal oedema and opacity, as the stroma fibres become separated (Saude, 1993). The expression and localization of the molecules that comprise the TJ complex are largely influenced by calcium dependent interactions with the cytoskeleton (Sugrue and Zieske, 1997). Preliminary studies were carried out to determine the optimal calcium concentration in the growth medium required to ensure the formation of a barrier to the passage of sodium fluorescein *in vitro* of 6% or less of the no-cell insert.

### **4.2.2 Materials and Methods**

Cascade Biologics™ Epilife growth media was prepared as section 2.1.7.1 except calcium chloride was added to produce final concentrations of 0, 60, 80, 100, 500 and 1000µM. J-HCET were harvested (section 2.2.3.3) and 500µl seeded into 0.45µm Nunc polycarbonate inserts at  $5 \times 10^5$  cells ml<sup>-1</sup> suspended in growth media containing 0, 60, 80, 100, 500 or 1000µM calcium. Two inserts received 500µl of medium containing 60µM calcium only and were referred to as the no-cell inserts. Inserts were placed into 24 well culture plates with 500µl of medium in the wells

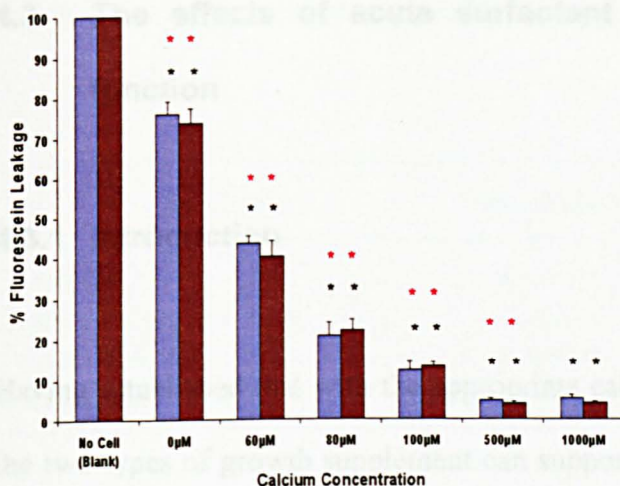
containing the same level of calcium as in the insert. Plates were maintained at 37°C, 5% CO<sub>2</sub> (v/v) in air for 72 hours without a change in medium. The leakage of fluorescein across the monolayer was determined (section 2.2.4.3) as was the protein content (section 2.2.4.1).

#### **4.2.3 Results**

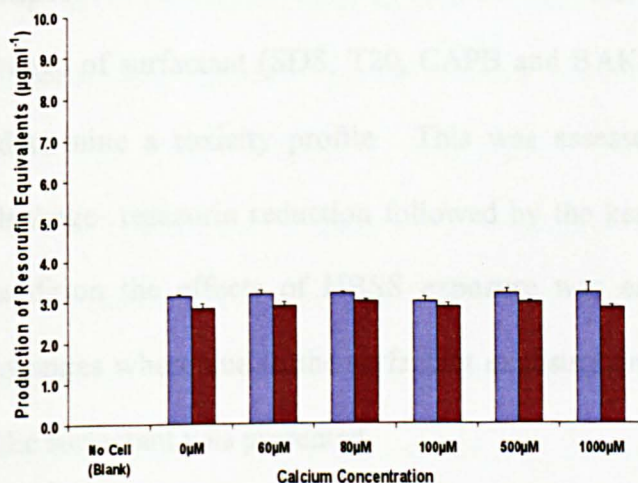
At calcium concentrations of 60µM to 100µM a significant reduction in fluorescein leakage was observed, compared to the no cell control (figure 4.04). The rates of resorufin production (figure 4.05) and total cell protein content (figure 4.06) were the same after 72 hours regardless of calcium concentration. To reduce the passage of fluorescein to approximately 6%, at 72 hours, a calcium concentration of 500µM or 1000µM was required.

#### **4.2.4 Conclusions**

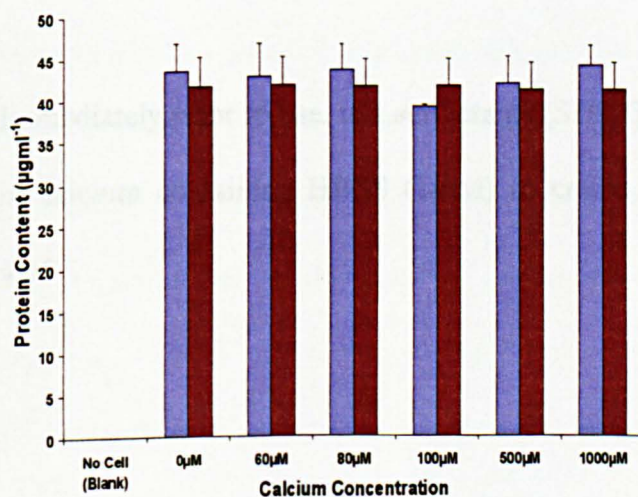
The degree of fluorescein leakage across J-HCET cells, cultured in medium with differing level of calcium, confirmed that the plant derived supplements do not significantly affect the capacity to form an effective barrier, i.e. the attainment of the leakage of 6% or (Clothier and Samson, 1996; Moore *et al.*, 2005). The production of resorufin, and total protein content of the J-HCET monolayers at all calcium concentrations remaining constant suggests that the alteration in fluorescein flux across the J-HCET monolayer was due to modifications of the adhesion molecule location or production, influenced by calcium concentration.



**Figure 4.04 :** Affect of medium calcium concentration on sodium fluorescein leakage across a confluent layer of J-HCET cultured in either HKGS ■ or HKGS-V2 ■ supplemented media. Where \* denotes a significant ( $P<0.05$ ) reduction in fluorescein leakage compared to no cell insert and \* denotes a significant reduction ( $P<0.05$ ) in leakage compared to previous calcium concentration. No significant difference was observed in the leakage between different supplements at identical calcium concentrations. (mean $\pm$ S.D, n=4)



**Figure 4.05 :** Affect of medium calcium concentration on resorufin production ( $\mu\text{gml}^{-1}$ ) of confluent J-HCET monolayers cultured in either HKGS ■ or HKGS-V2 ■ supplemented media containing 0, 60, 80, 100, 500 or 1000 $\mu\text{M}$  calcium chloride. (mean $\pm$ S.D, n=4)



**Figure 4.06 :** Affect of medium calcium concentration on protein content of confluent J-HCET monolayers cultured in either HKGS ■ or HKGS-V2 ■ supplemented media containing 0, 60, 80, 100, 500 or 1000 $\mu\text{M}$  calcium chloride. (mean $\pm$ S.D, n=4)

### **4.3 The effects of acute surfactant exposure on J-HCET barrier function**

#### **4.3.1 Introduction**

Having established that with the appropriate calcium levels in the medium either of the two types of growth supplement can support effective barrier formation, studies were carried out to determine the amount of damage caused by acute surfactant exposure to J-HCET monolayers. Cultured in polycarbonate tissue culture inserts, a range of surfactant (SDS, T20, CAPB and BAK) concentrations were employed, to determine a toxicity profile. This was assessed using the combined fluorescein leakage- resazurin reduction followed by the kenacid blue total protein assays. In addition the effects of HBSS exposure was examined to ensure any observable changes were due to the surfactant exposure and not the delivery vehicle in which the surfactant was presented.

#### **4.3.2 Materials and Methods**

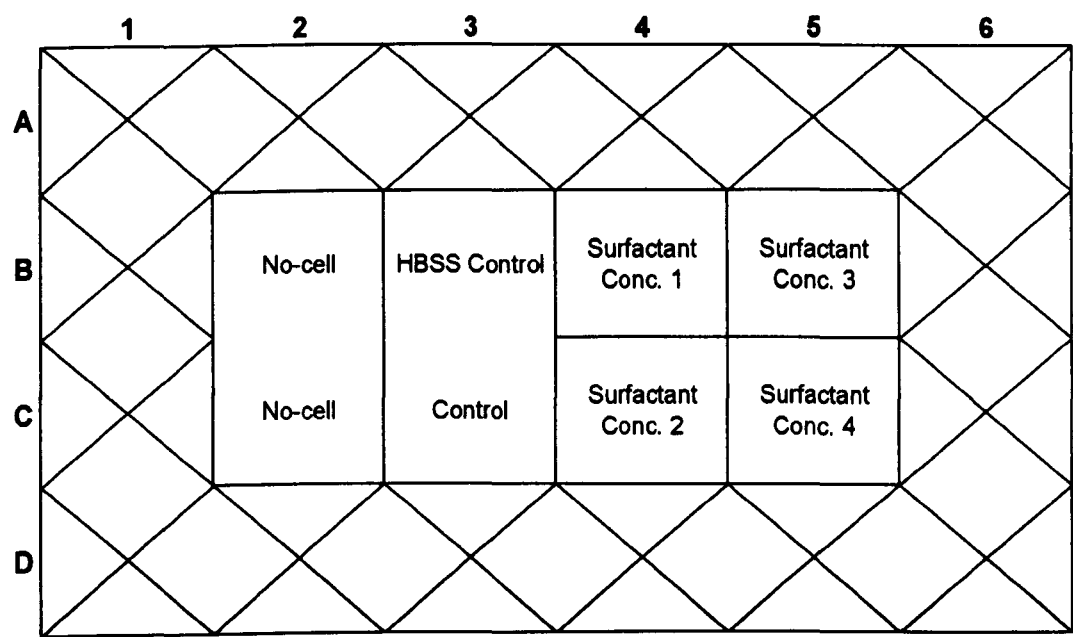
Immediately prior to use, test surfactants (SDS, T20, CAPB or BAK) were dissolved in calcium containing HBSS (1mM) to create concentrations as outlined in table 4.01.

**Table 4.01 : Surfactant toxicity profile concentration ranges.**

Surfactant	Ionic Status	Concentration(mgml <sup>-1</sup> ) in HBSS			
Tween 20	Non-ionic	400	350	300	250
Sodium Dodecyl Sulphate	Anionic	0.80	0.40	0.20	0.10
Cocamidopropylbentaine	Amphoteric	0.50	0.40	0.30	0.20
Benzalkonium Chloride	Cationic	0.125	0.1	0.075	0.05

J-HCET were seeded, (500µl), into 2 Nunc Polycarbonate inserts at  $5 \times 10^5$  cells ml<sup>-1</sup> suspended in Epilife™ growth medium containing subcytotoxic concentrations of SDS, T20, CAPB or BAK. Growth medium (500µl) was added to the outer 16 wells to prevent excessive evaporation. Two inserts received 500µl of medium alone and were referred to as the no-cell inserts. Inserts were placed into 24 well plates with an additional 500µl of corresponding medium in the wells (figure 4.07). Plates were maintained at 37°C, 5% CO<sub>2</sub> (v/v) in air. For each surfactant 5 replicate plates were established. After 72 hours in culture the medium was aspirated from one plate and the combined fluorescein leakage- resazurin reduction followed by the total protein kenacid blue assays performed as previously described (sections 2.2.4.4 and 2.2.4.1). From the remaining plates medium was aspirated from the inserts and 400µl of one of each of the acute surfactant concentrations (table 4.01) applied for 60 seconds. The surfactant was removed and the monolayers washed with 200µl of HBSS (2 x 60 seconds). HBSS was aspirated and 500µl of cell culture medium (pre-warmed to 37°C) applied. The combined fluorescein leakage- resazurin reduction and kenacid blue assays were performed on one plate per time point, every 24 hours over 96 hours.

**Figure 4.07 :** Polycarbonate insert seeding template for acute surfactant exposure.



Where No-cell denotes 500µl media only, control represents J-HCET cultured in normal growth media and HBSS control denotes J-HCET exposed to HBSS only.

**4.3.3 Results**

**4.3.3.1Sodium dodecyl sulphate acute toxicity profile**

Exposure to SDS at a concentration of 0.1mgml<sup>-1</sup> caused the leakage of fluorescein across the J-HCET monolayer to be significantly (P<0.05) increased at 24 hours post exposure, followed by subsequent full recovery of barrier function by 96 hours (figure 4.08). SDS concentrations of 0.2, 0.4 and 0.8mgml<sup>-1</sup> caused a concentration dependent increase in leakage at 24 hours post exposure and subsequently partial but incomplete recovery by 96 hours (figure 4.08). The cell activity following exposure to 0.1, 0.2, 0.4 and 0.8mgml<sup>-1</sup> SDS significantly decreased at 24 hours with

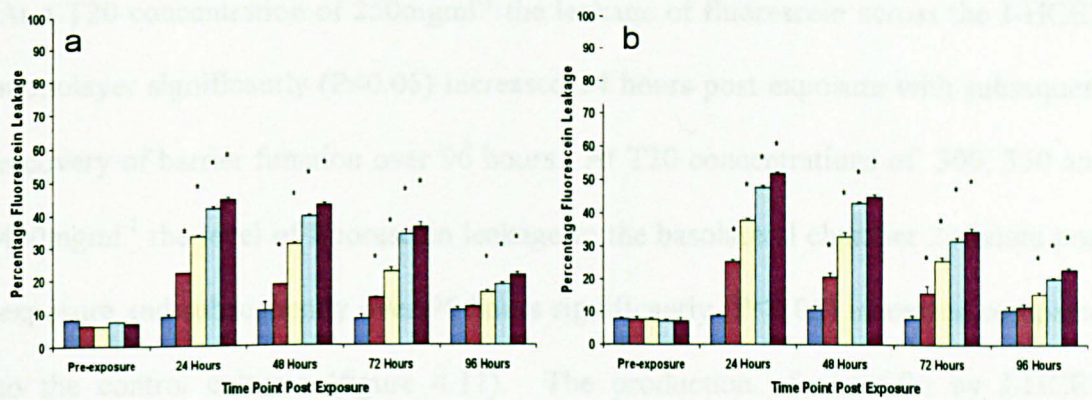
concentration dependent recovery by 72 hours (figure 4.09). The total cell number, i.e. the protein content, of the J-HCET monolayers exposed to 0.4 and 0.8mgml<sup>-1</sup> SDS was significantly decreased at 24 hours with subsequent complete recovery by 48 hours (figure 4.10).

#### **4.3.3.1.1 Conclusion**

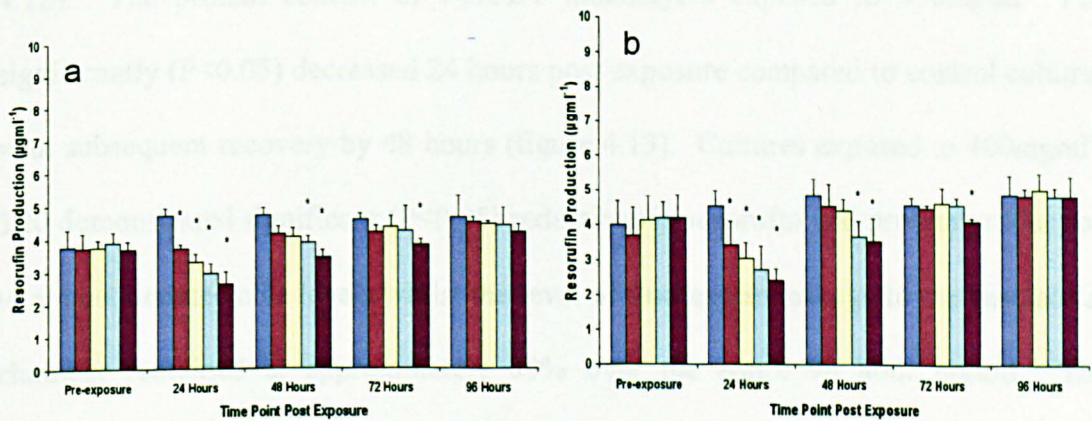
Whilst at 24 hours the leakage, cell activity and cell number show a concentration dependent reduction, at subsequent times the leakage was affected more than the cell activity whilst the cell number was unaffected. The toxicity profiles for fluorescein leakage, resorufin production and protein content were comparable for J-HCET cultured in growth media containing either HKGS (figure 4.08a) or HKGS-V2 (figure 4.08b) supplements i.e. the different growth supplements do not affect recovery from injury.



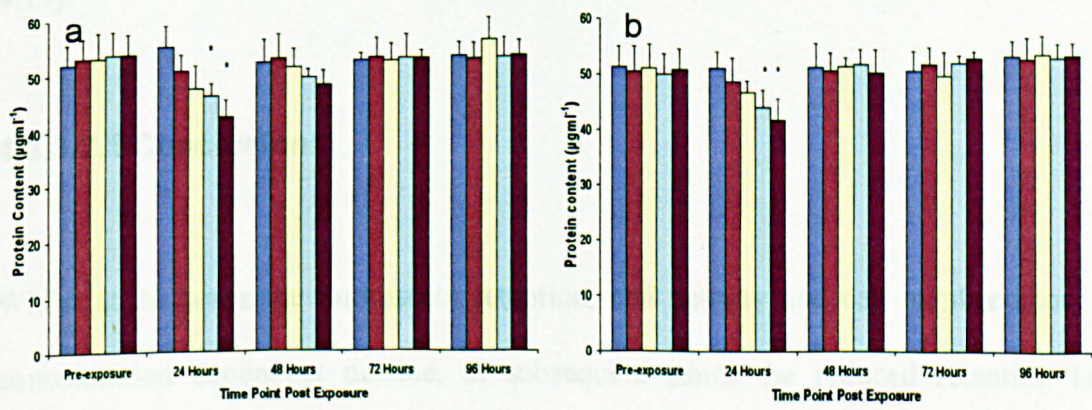
**Figure 4.08 a/b** : fluorescein leakage across J-HCET monolayers cultured in (a) HKGS or (b) HKGS-V2 supplemented media over 96 hours subsequent to acute (60 seconds) sodium dodecyl sulphate exposure ■ Control, ■ 0.1mgml<sup>-1</sup>, ■ 0.2mgml<sup>-1</sup>, ■ 0.4mgml<sup>-1</sup>, and ■ 0.8mgml<sup>-1</sup> where \* denotes a significant (P<0.05) decrease in resorufin production compared to control cultures (mean±S.D., n=3).



**Figure 4.09 a/b** : Resorufin production (µgml<sup>-1</sup>) of J-HCET monolayers cultured in (a) HKGS or (b) HKGS-V2 over 96 hours subsequent to acute (60 seconds) sodium dodecyl sulphate exposure ■ Control, ■ 0.1mgml<sup>-1</sup>, ■ 0.2mgml<sup>-1</sup>, ■ 0.4mgml<sup>-1</sup>, and ■ 0.8mgml<sup>-1</sup> where \* denotes a significant (P<0.05) increase in leakage compared to control cultures (mean±S.D., n=3).



**Figure 4.10 a/b** : Protein content (µgml<sup>-1</sup>) of J-HCET monolayers cultured in (a) HKGS or (b) HKGS-V2 over 96 hours subsequent to acute (60 seconds) sodium dodecyl sulphate exposure ■ Control, ■ 0.1mgml<sup>-1</sup>, ■ 0.2mgml<sup>-1</sup>, ■ 0.4mgml<sup>-1</sup>, and ■ 0.8mgml<sup>-1</sup> where \* denotes a significant (P<0.05) increase in protein content compared to control cultures (mean±S.D., n=3).





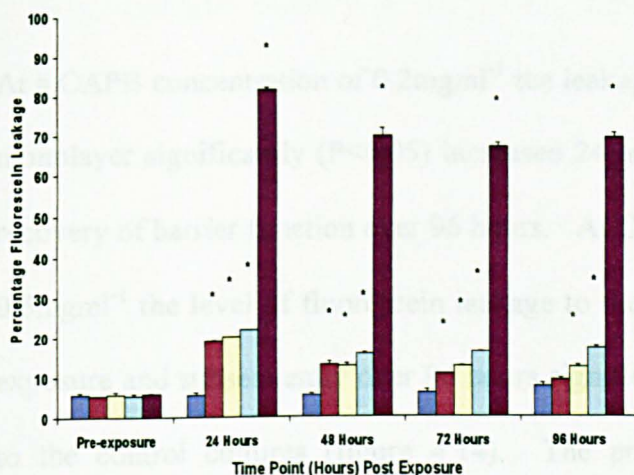
#### **4.3.3.2 Tween 20 acute toxicity profile**

At a T20 concentration of  $250\text{mgml}^{-1}$  the leakage of fluorescein across the J-HCET monolayer significantly ( $P<0.05$ ) increased 24 hours post exposure with subsequent recovery of barrier function over 96 hours. At T20 concentrations of 300, 350 and  $400\text{mgml}^{-1}$  the level of fluorescein leakage to the basolateral chamber 24 hours post exposure and subsequently over 96 hours significantly ( $P<0.05$ ) increased compared to the control cultures (figure 4.11). The production of resorufin by J-HCET monolayers exposed to 250, 300 and  $350\text{mgml}^{-1}$  T20 significantly ( $P<0.05$ ) decreased 24 hours post exposure with subsequent recovery by 72 hours (figure 4.12). The protein content of J-HCET monolayers exposed to  $350\text{mgml}^{-1}$  T20 significantly ( $P<0.05$ ) decreased 24 hours post exposure compared to control cultures with subsequent recovery by 48 hours (figure 4.13). Cultures exposed to  $400\text{mgml}^{-1}$  T20 demonstrated significant ( $P<0.05$ ) reductions in resorufin and protein production to almost undetectable levels whilst the level of fluorescein leakage to the basolateral chamber remained at approximately 80% over the entire 96 hour period. The toxicity profiles for fluorescein leakage, resorufin production and protein content were comparable for J-HCET cultured in growth media containing either HKGS or HKGS-V2 supplements, hence only those for HKGS were shown (figure 4.11 to 4.13).

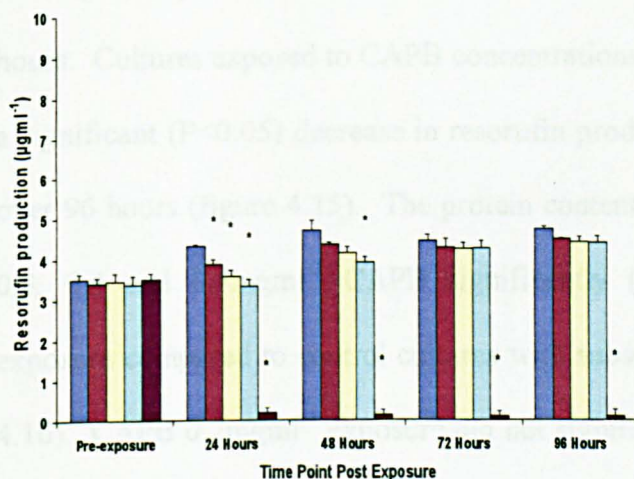
##### **4.3.3.2.1 Conclusions**

Whilst at 24 hours the fluorescein retention, cell activity and cell number show a concentration dependent decline, at subsequent times the reduced retention i.e.

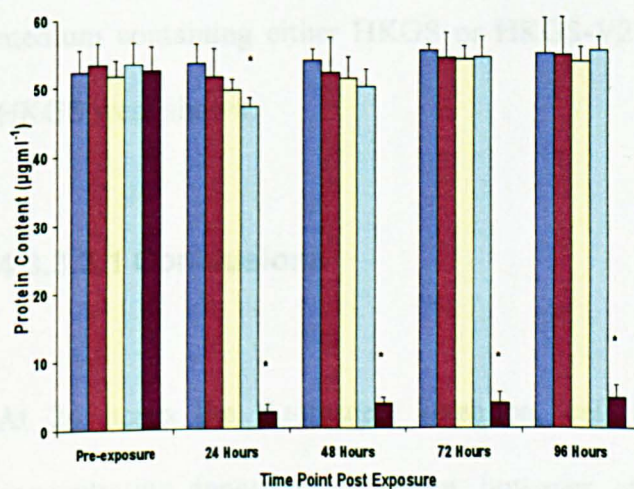
increased leakage was affected more than the cell activity whilst the cell number was unaffected. The nature of the medium supplement did not affect the toxicity profiles, i.e. fluorescein leakage, resorufin production and protein content. The non-ionic surfactant did not interact with the cells differently as a result of choice of supplement.



**Figure 4.11** : Leakage of fluorescein across J-HCET monolayers over 96 hours subsequent to acute (60 seconds) tween 20 exposure ■ Control, ■ 250mgml<sup>-1</sup>, ■ 300mgml<sup>-1</sup>, ■ 350mgml<sup>-1</sup>, and ■ 400mgml<sup>-1</sup> where \* denotes a significant (P<0.05) increase in fluorescein leakage compared to control cultures (mean±S.D., n=3).



**Figure 4.12** : Production of resorufin equivalents (μgml<sup>-1</sup>) of J-HCET monolayers over 96 hours subsequent to acute (60 seconds) tween 20 exposure ■ Control, ■ 250mgml<sup>-1</sup>, ■ 300mgml<sup>-1</sup>, ■ 350mgml<sup>-1</sup>, and ■ 400mgml<sup>-1</sup> where \* denotes a significant (P<0.05) decrease in resorufin production compared to control cultures (mean±S.D., n=3).



**Figure 4.13** : Protein content (μgml<sup>-1</sup>) of J-HCET monolayers over 96 hours subsequent to acute (60 seconds) tween 20 exposure ■ Control, ■ 250mgml<sup>-1</sup>, ■ 300mgml<sup>-1</sup>, ■ 350mgml<sup>-1</sup>, and ■ 400mgml<sup>-1</sup> where \* denotes a significant (P<0.05) decrease in protein content compared to control cultures (mean±S.D., n=3).

#### **4.3.3.3 Cocamidopropylbetaine acute toxicity profile**

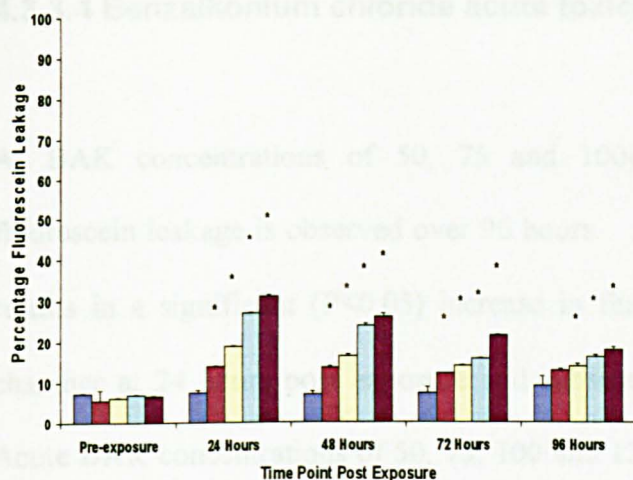
At a CAPB concentration of  $0.2\text{mgml}^{-1}$  the leakage of fluorescein across the J-HCET monolayer significantly ( $P<0.05$ ) increased 24 hours post exposure with subsequent recovery of barrier function over 96 hours. At CAPB concentrations of 0.3, 0.4 and  $0.5\text{mgml}^{-1}$  the level of fluorescein leakage to the basolateral chamber 24 hours post exposure and subsequently over 96 hours significantly ( $P<0.05$ ) increased compared to the control cultures (figure 4.14). The production of resorufin by J-HCET monolayers exposed to 0.2 and  $0.3\text{mgml}^{-1}$  CAPB significantly ( $P<0.05$ ) decreased 24 hours post exposure compared to control cultures with subsequent recovery by 96 hours. Cultures exposed to CAPB concentrations of 0.4 and  $0.5\text{mgml}^{-1}$  demonstrated a significant ( $P<0.05$ ) decrease in resorufin production with no subsequent recovery over 96 hours (figure 4.15). The protein content of J-HCET monolayers exposed to 0.3, 0.4 and  $0.5\text{mgml}^{-1}$  CAPB significantly ( $P<0.05$ ) decreased 24 hours post exposure compared to control cultures with subsequent recovery by 96 hours (figure 4.16). CAPB  $0.2\text{mgml}^{-1}$  exposure did not significantly alter the protein content of J-HCET monolayers. The toxicity profiles for fluorescein leakage, resorufin production and protein content were comparable for J-HCET cultured in growth medium containing either HKGS or HKGS-V2 supplements, hence only those for HKGS were shown.

##### **4.3.3.3.1 Conclusions**

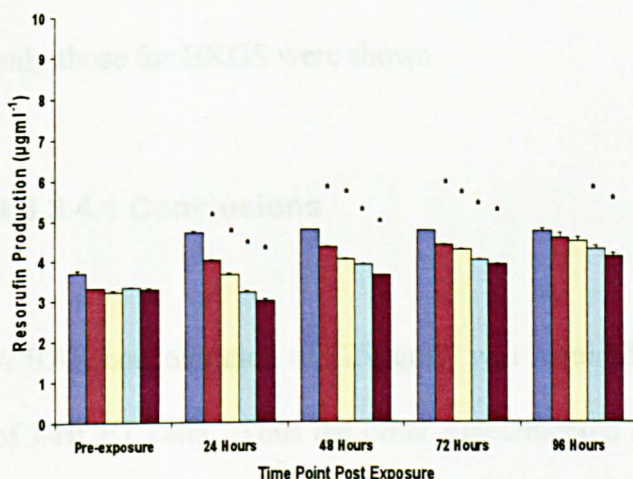
At 24 hours the fluorescein retention, cell activity and cell number show a concentration dependent reduction, however, at subsequent times the leakage and

activity were affected more than protein content. The toxicity profiles for fluorescein leakage, resorufin production and protein content were comparable for J-HCET cultured in growth media containing either HKGS or HKGS-V2 supplements.

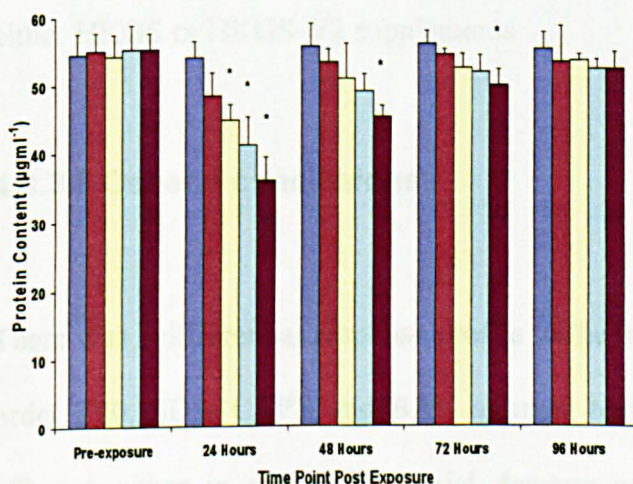




**Figure 4.14** : Leakage of fluorescein across J-HCET monolayers over 96 hours subsequent to acute (60 seconds) cocamidopropylbetaine exposure Control, 0.2mgml<sup>-1</sup>, 0.3mgml<sup>-1</sup>, 0.4mgml<sup>-1</sup>, and 0.5mgml<sup>-1</sup> where \* denotes a significant (P<0.05) increase in fluorescein leakage compared to control cultures (mean±S.D., n=3).



**Figure 4.15** : Production of resorufin equivalents (µgml<sup>-1</sup>) of J-HCET monolayers over 96 hours subsequent to acute (60 seconds) cocamidopropylbetaine exposure Control, 0.2mgml<sup>-1</sup>, 0.3mgml<sup>-1</sup>, 0.4mgml<sup>-1</sup>, and 0.5mgml<sup>-1</sup> where \* denotes a significant (P<0.05) decrease in resorufin production compared to control cultures (mean±S.D., n=3).



**Figure 4.16** : Protein content (µgml<sup>-1</sup>) of J-HCET monolayers over 96 hours subsequent to acute (60 seconds) cocamidopropylbetaine exposure Control, 0.2mgml<sup>-1</sup>, 0.3mgml<sup>-1</sup>, 0.4mgml<sup>-1</sup>, and 0.5mgml<sup>-1</sup> where \* denotes a significant (P<0.05) decrease in protein content compared to control cultures (mean±S.D., n=3).

#### **4.3.3.4 Benzalkonium chloride acute toxicity profile**

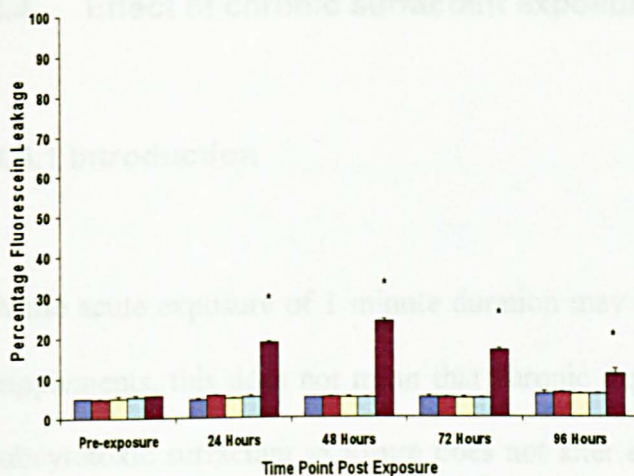
At BAK concentrations of 50, 75 and 100 $\mu\text{gml}^{-1}$  no significant increase in fluorescein leakage is observed over 96 hours. A BAK concentration of 125 $\mu\text{gml}^{-1}$  results in a significant ( $P<0.05$ ) increase in fluorescein leakage to the basolateral chamber at 24 hours post exposure and subsequently over 96 hours (figure 4.17). Acute BAK concentrations of 50, 75, 100 and 125 $\mu\text{gml}^{-1}$  have no significant effects on either resorufin production or total protein content (figures 4.18 and 4.19) when J-HCET were cultured in medium containing HKGS or HKGS-V2 supplements, hence only those for HKGS were shown.

##### **4.3.3.4.1 Conclusions**

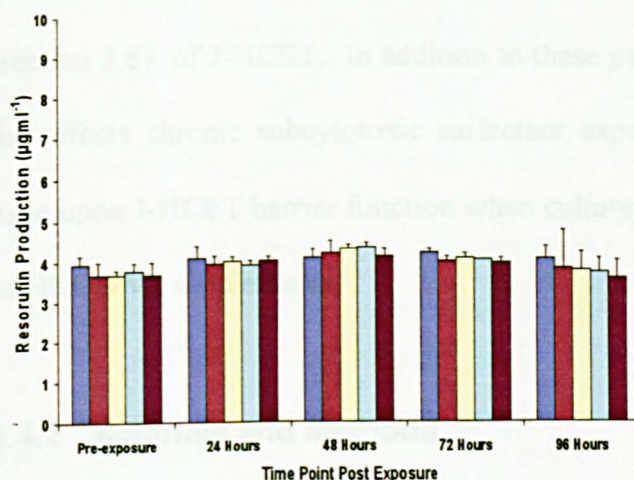
A BAK concentration of 125 $\mu\text{gml}^{-1}$  was required to compromise the barrier function of J-HCET cells. Thus the other supplemented medium was tested for comparative effects. The toxicity profiles for fluorescein leakage, resorufin production and protein content were comparable for J-HCET cultured in growth media containing either HKGS or HKGS-V2 supplements.

##### **4.3.3.5 General conclusions**

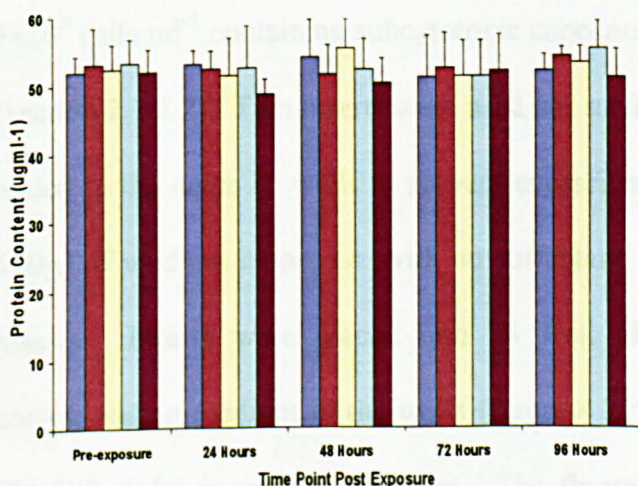
There was a differential response profile to the different classes of surfactants, in the order T20, SDS, CAPB and BAK as most toxic. The cultures response was not affected either in respect of initial damage or duration or completeness of the recovery as a result of the growth supplement employed.



**Figure 4.17** : Leakage of fluorescein across J-HCET monolayers over 96 hours subsequent to acute (60 seconds) benzalkonium chloride  $\blacksquare$  Control,  $\blacksquare$  50 $\mu\text{gml}^{-1}$ ,  $\blacksquare$  75 $\mu\text{gml}^{-1}$ ,  $\blacksquare$  100 $\mu\text{gml}^{-1}$ , and  $\blacksquare$  125 $\mu\text{gml}^{-1}$  where \* denotes a significant ( $P<0.05$ ) increase in fluorescein leakage compared to control cultures (mean $\pm$ S.D.,  $n=3$ ).



**Figure 4.18** : Production of resorufin equivalents ( $\mu\text{gml}^{-1}$ ) of J-HCET monolayers over 96 hours subsequent to acute (60 seconds) benzalkonium chloride  $\blacksquare$  Control,  $\blacksquare$  50 $\mu\text{gml}^{-1}$ ,  $\blacksquare$  75 $\mu\text{gml}^{-1}$ ,  $\blacksquare$  100 $\mu\text{gml}^{-1}$ , and  $\blacksquare$  125 $\mu\text{gml}^{-1}$  where \* denotes a significant ( $P<0.05$ ) decrease in resorufin production compared to control cultures (mean $\pm$ S.D.,  $n=3$ ).



**Figure 4.19** : Protein content ( $\mu\text{gml}^{-1}$ ) of J-HCET monolayers over 96 hours subsequent to acute (60 seconds) benzalkonium chloride  $\blacksquare$  Control,  $\blacksquare$  50 $\mu\text{gml}^{-1}$ ,  $\blacksquare$  75 $\mu\text{gml}^{-1}$ ,  $\blacksquare$  100 $\mu\text{gml}^{-1}$ , and  $\blacksquare$  125 $\mu\text{gml}^{-1}$  where \* denotes a significant ( $P<0.05$ ) decrease in protein content compared to control cultures (mean $\pm$ S.D.,  $n=3$ ).



## **4.4 Effect of chronic surfactant exposure on J-HCET barrier function**

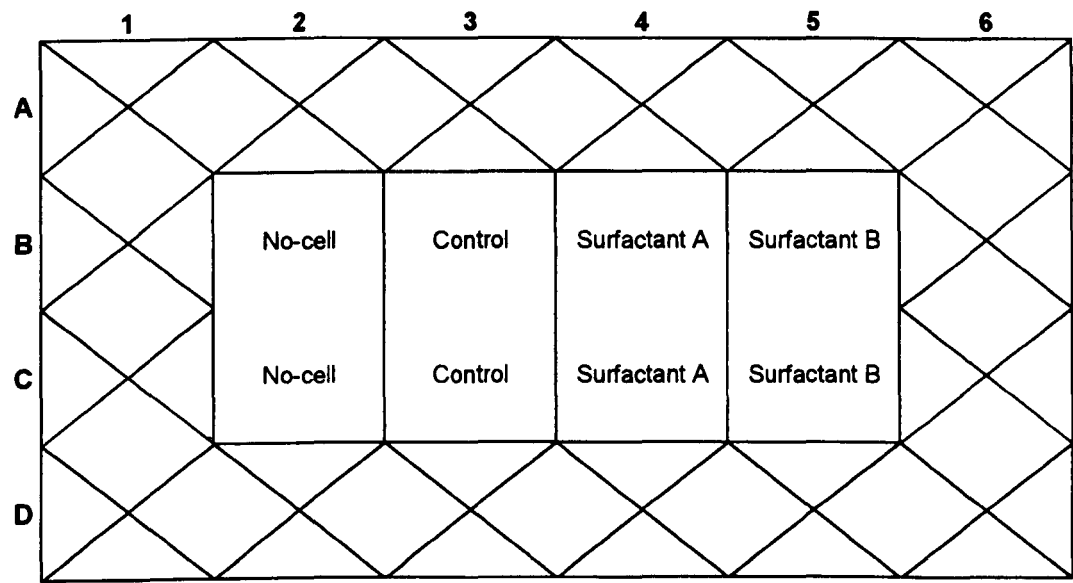
### **4.4.1 Introduction**

While acute exposure of 1 minute duration may not be affected by growth medium supplements, this does not mean that chronic exposure is also unaffected. Chronic subcytotoxic surfactant exposure does not alter either the population doubling time (section 3.5) or the production of resorufin, neutral red uptake and protein content (section 3.6) of J-HCET. In addition to these parameters it was necessary to assess the effects chronic subcytotoxic surfactant exposure (SDS, T20, CAPB or BAK) have upon J-HCET barrier function when cultured in media containing either HKGS or HKGS-V2 supplements.

### **4.4.2 Materials and Methods**

J-HCET were seeded, in 500 $\mu$ l Epilife growth media, into polycarbonate inserts at  $5 \times 10^5$  cells ml<sup>-1</sup> containing subcytotoxic concentrations of SDS, T20, CAPB or BAK (section 2.1.7.2). Two inserts were used per surfactant. Growth medium (500 $\mu$ l) was added to the outer 16 wells to prevent excessive evaporation. Two inserts received 500 $\mu$ l of medium alone, i.e. with no surfactant, and were referred to as the no-cell inserts. Inserts were placed into 24 well plates with an additional 500 $\mu$ l of corresponding medium in the wells (figure 4.20). Plates were maintained at 37°C, 5% CO<sub>2</sub> (v/v) in air for 72 hours. The fluorescein leakage (section 2.2.4.3) and kenacid blue (section 2.2.4.1) assays were performed every 72 hours upto 504 hours.

**Figure 4.20 :** Polycarbonate insert seeding template for chronic surfactant exposure.



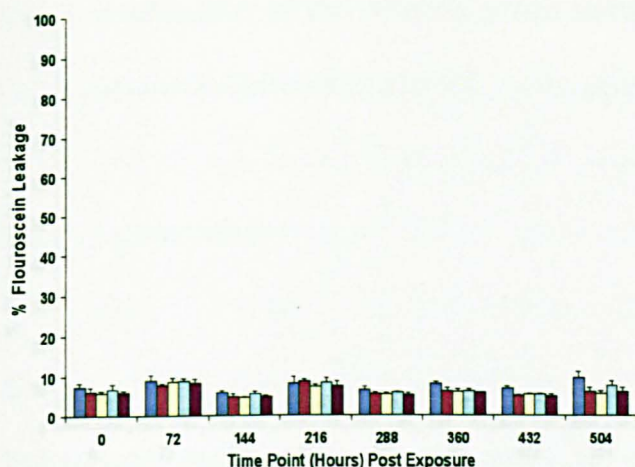
*Where No-cell denotes 500 $\mu$ l media only, control represents J-HCET cultured in normal growth media and Surfactant A/B denotes J-HCET cultured in media containing SDS, T20, CAPB or BAK.*

### 4.4.3 Results

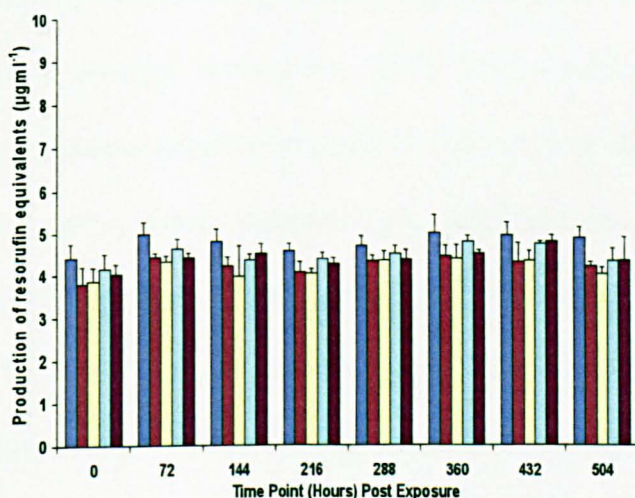
No significant alterations in fluorescein leakage were observed compared to control cultures when J-HCET were cultured in growth media (with either HKGS (figure 4.21) or HKGS-V2 (figure 4.24) supplements) containing SDS (4 $\mu$ gml<sup>-1</sup>), T20 (25 $\mu$ gml<sup>-1</sup>), CAPB (3 $\mu$ gml<sup>-1</sup>) or BAK (0.0025 $\mu$ gml<sup>-1</sup>). The degree of fluorescein leakage across the monolayer when cultured in medium containing subcytotoxic surfactant concentrations confirms that the surfactants do not significantly affect the capacity to produce an effective barrier (figures 4.21 and 4.24). In addition no significant alterations in either the production of resorufin (figures 4.22 and 4.25) or total protein content of the J-HCET monolayers was observed compared to the control cultures regardless of medium composition (figures 4.23 and 4.26).

#### **4.4.4 Conclusions**

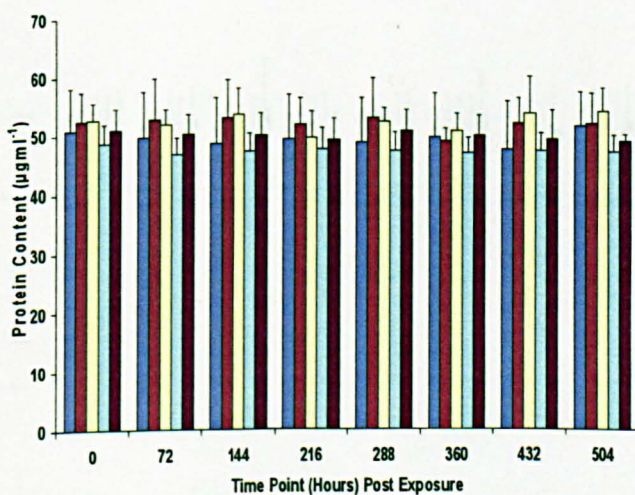
Subcytotoxic surfactant exposure of SDS;  $4\mu\text{gml}^{-1}$ , T20;  $25\mu\text{gml}^{-1}$ , CAPB  $2.99\mu\text{gml}^{-1}$  or BAK;  $0.0025\mu\text{gml}^{-1}$ ) have no significant effects on barrier function, total cell protein or activity over 21 days (504 hours), regardless of supplement (HKGS or HKGS-V2) employed. Even at 504 hours no indications of pending adverse reactions were noted.



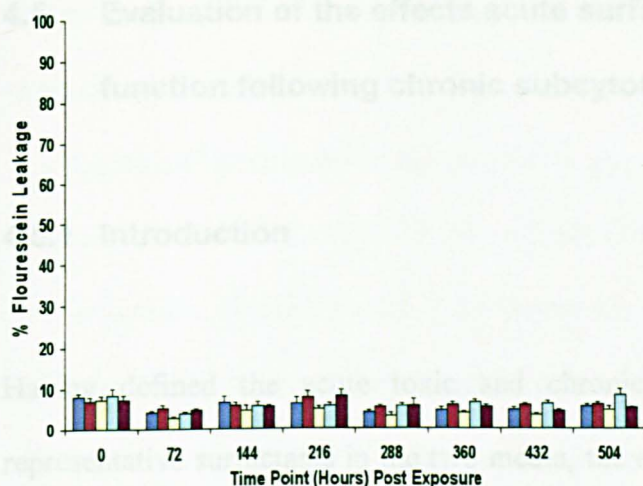
**Figure 4.21** : Barrier integrity of J-HCET monolayers cultured in HKGS supplemented growth media ■ containing ■ sodium dodecyl sulphate ( $4\mu\text{gml}^{-1}$ ), ■ tween 20 ( $25\mu\text{gml}^{-1}$ ), ■ cocamidopropylbetaine ( $3\mu\text{gml}^{-1}$ ) or ■ benzalkonium chloride ( $0.0025\mu\text{gml}^{-1}$ ). (mean $\pm$ S.D., n=3).



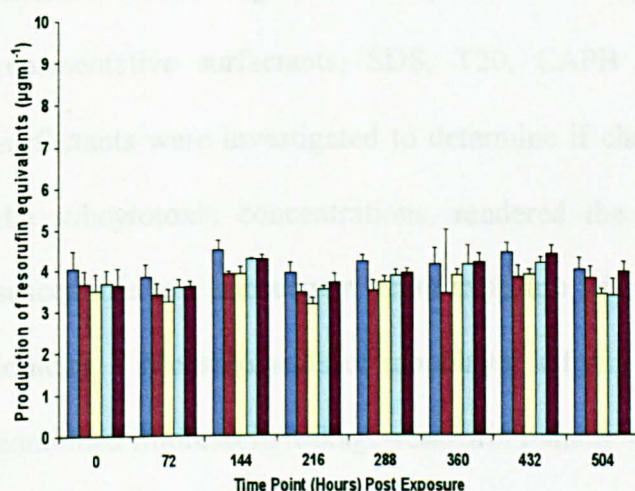
**Figure 4.22** : Resorufin production of J-HCET monolayers cultured in HKGS supplemented growth media ■ containing ■ sodium dodecyl sulphate ( $4\mu\text{gml}^{-1}$ ), ■ tween 20 ( $25\mu\text{gml}^{-1}$ ), ■ cocamidopropylbetaine ( $3\mu\text{gml}^{-1}$ ) or ■ benzalkonium chloride ( $0.0025\mu\text{gml}^{-1}$ ). (mean $\pm$ S.D., n=3).



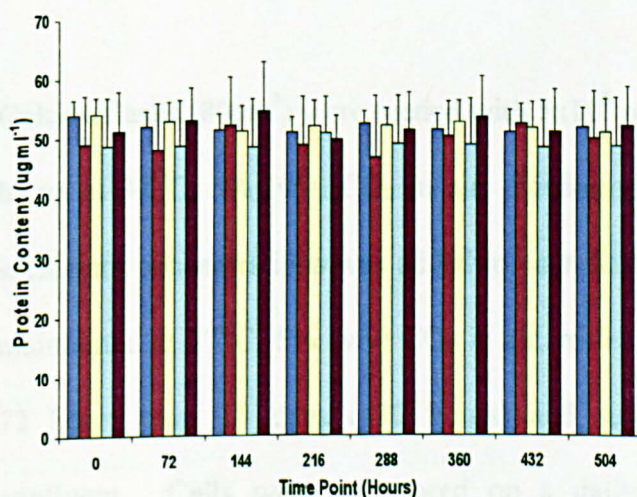
**Figure 4.23** : Protein content of J-HCET monolayers cultured in HKGS supplemented growth media ■ containing ■ sodium dodecyl sulphate ( $4\mu\text{gml}^{-1}$ ), ■ tween 20 ( $25\mu\text{gml}^{-1}$ ), ■ cocamidopropylbetaine ( $3\mu\text{gml}^{-1}$ ) or ■ benzalkonium chloride ( $0.0025\mu\text{gml}^{-1}$ ). (mean $\pm$ S.D., n=3).



**Figure 4.24** : Barrier integrity of J-HCET monolayers cultured in HKGS-V2 supplemented growth media containing sodium dodecyl sulphate ( $4\mu\text{gml}^{-1}$ ), tween 20 ( $25\mu\text{gml}^{-1}$ ), cocamidopropylbetaine ( $3\mu\text{gml}^{-1}$ ) or benzalkonium chloride ( $0.0025\mu\text{gml}^{-1}$ ). (mean $\pm$ S.D., n=3).



**Figure 4.25** : Resorufin production of J-HCET monolayers cultured in HKGS-V2 supplemented growth media containing sodium dodecyl sulphate ( $4\mu\text{gml}^{-1}$ ), tween 20 ( $25\mu\text{gml}^{-1}$ ), cocamidopropylbetaine ( $3\mu\text{gml}^{-1}$ ) or benzalkonium chloride ( $0.0025\mu\text{gml}^{-1}$ ). (mean $\pm$ S.D., n=3).



**Figure 4.26** : Protein content of J-HCET monolayers cultured in HKGS-V2 supplemented growth media containing sodium dodecyl sulphate ( $4\mu\text{gml}^{-1}$ ), tween 20 ( $25\mu\text{gml}^{-1}$ ), cocamidopropylbetaine ( $3\mu\text{gml}^{-1}$ ) or benzalkonium chloride ( $0.0025\mu\text{gml}^{-1}$ ). (mean $\pm$ S.D., n=3).



## **4.5 Evaluation of the effects acute surfactant exposure has on barrier function following chronic subcytotoxic exposure**

### **4.5.1 Introduction**

Having defined the acute toxic and chronic non-toxic concentrations of the representative surfactants in the two media, the effects of chronic exposure on acute response can be investigated. Initially the viability of the J-HCET monolayers was monitored following chronic exposure to subcytotoxic concentrations of the four representative surfactants; SDS, T20, CAPB and BAK. Interactions between surfactants were investigated to determine if chronic exposure to one surfactant, at the subcytotoxic concentrations, rendered the J-HCET monolayer more or less susceptible to a subsequent acute exposure with the same or surfactant of different ionicity. Measurements of monolayer activity and integrity were made using the combined fluorescein leakage-resazurin reduction and kenacid blue assays.

### **4.5.2 Materials and Methods**

Culture flasks (80cm<sup>3</sup>) were seeded with  $5 \times 10^5$  of J-HCET cells and incubated for 24 hours at 37°C, 5% (v/v) CO<sub>2</sub> in air. Medium or medium containing subcytotoxic surfactant concentrations was added to each flask where appropriate. Cultures were maintained at 37°C, 5% (v/v) CO<sub>2</sub> in air, re-fed with the appropriate medium every 72 hours over 18 days (432 hours) and subcultured once cells were 70-80% confluent. Cells were monitored on a daily basis at low magnification (x10 objective) to ascertain the level of cell growth, cell morphology and to check for

contamination. Following the 18 day exposure, J-HCET cells were harvested from large culture flasks (section 2.2.3.3) and 500 $\mu$ l seeded into Polycarbonate inserts at  $5 \times 10^5$  cells  $\text{ml}^{-1}$  suspended in the respective growth medium containing subcytotoxic concentrations of SDS, T20, CAPB or BAK (section 2.1.7.2) four replicates inserts per surfactant. Growth media (500 $\mu$ l) was added to the outer 16 wells to prevent excessive evaporation. Two inserts received 500 $\mu$ l of medium alone and were referred to as the no-cell inserts. Inserts were placed into 24 well plates with an additional 500 $\mu$ l of corresponding medium in the wells and incubated at 37°C, 5% CO<sub>2</sub> (v/v) in air for 72 hours. For each surfactant 5 replicate plates were established. Medium was aspirated from one plate and the combined fluorescein leakage-resazurin reduction (section 2.2.4.3) and total protein kenacid blue (section 2.2.4.1) assays performed. From the remaining plates medium was aspirated and 400 $\mu$ l of each of the acute surfactant concentrations (table 4.01) applied for 60 seconds. This was then removed from the inserts via aspiration, and the monolayers washed with 200 $\mu$ l of HBSS (2 x 60 seconds). HBSS was aspirated and 500 $\mu$ l of cell culture media (pre-warmed to 37°C) applied. The combined fluorescein leakage-resazurin reduction and kenacid blue assays were performed every 24 hours over 96 hours.

### **4.5.3 Results**

#### **4.5.3.1 Effects of surfactant pre-exposure on acute SDS (800 $\mu\text{gml}^{-1}$ ) toxicity**

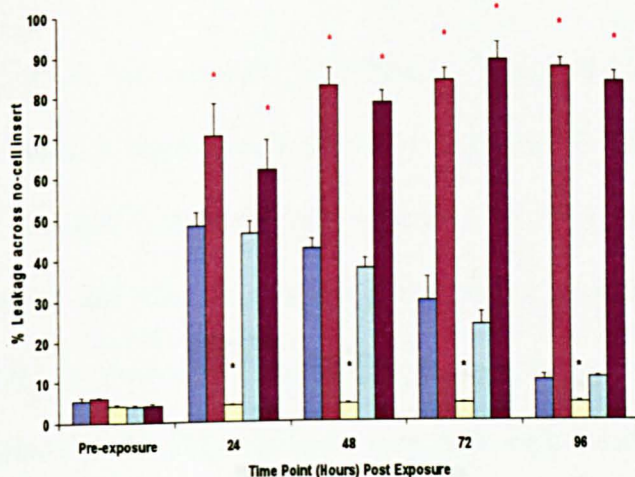
Chronic subcytotoxic SDS (4 $\mu\text{gml}^{-1}$ ) and BAK (0.0025 $\mu\text{gml}^{-1}$ ) exposure significantly ( $P < 0.05$ ) increased the toxicity of a subsequent acute SDS (800 $\mu\text{gml}^{-1}$ ) exposure, demonstrated by an increase in fluorescein leakage (figure 4.27) and reductions in the production of resorufin (figure 4.28) and protein content (figure



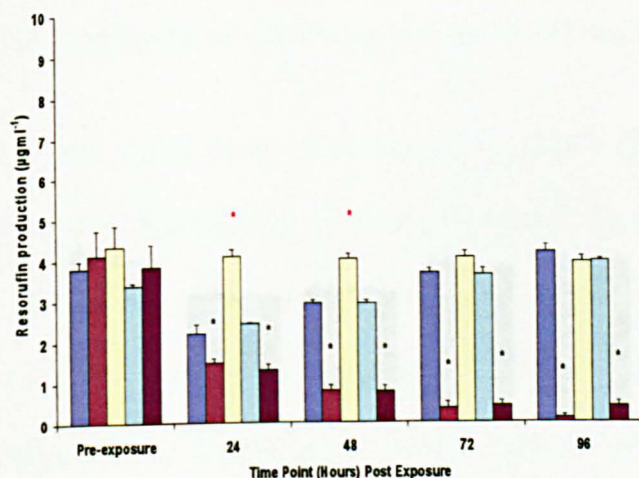
4.29) compared to control cultures over 96 hours. T20 ( $25\mu\text{gml}^{-1}$ ) pre-exposure significantly ( $P<0.05$ ) decreased the toxicity of subsequent acute exposures of SDS ( $800\mu\text{gml}^{-1}$ ) whilst CAPB ( $3\mu\text{gml}^{-1}$ ) pre-exposure did not significantly modulate the toxicity of subsequent acute SDS exposures.

#### **4.5.3.2 Effects of surfactant pre-exposure on acute T20 ( $300\text{mgml}^{-1}$ ) toxicity**

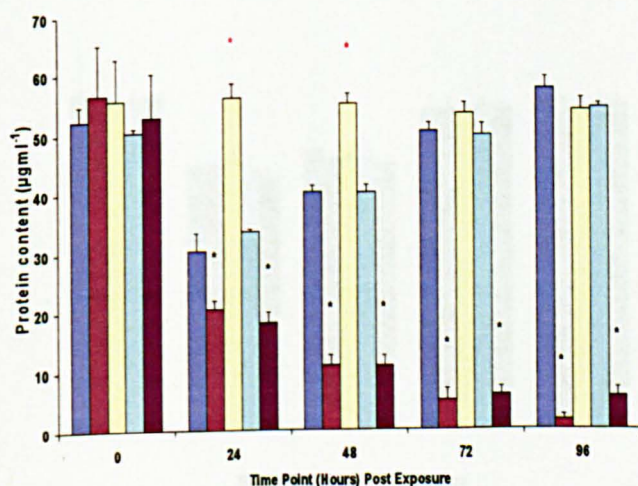
SDS ( $4\mu\text{gml}^{-1}$ ), T20 ( $25\mu\text{gml}^{-1}$ ), CAPB ( $3\mu\text{gml}^{-1}$ ) or BAK ( $0.0025\mu\text{gml}^{-1}$ ) pre-exposure did not significantly modulate the toxicity of subsequent acute T20 ( $300\text{mgml}^{-1}$ ) exposures. Demonstrated by fluorescein leakage (figure 4.30), resorufin production (figure 4.31) and protein content (figure 4.32) levels equivalent to the control over 96 hours.



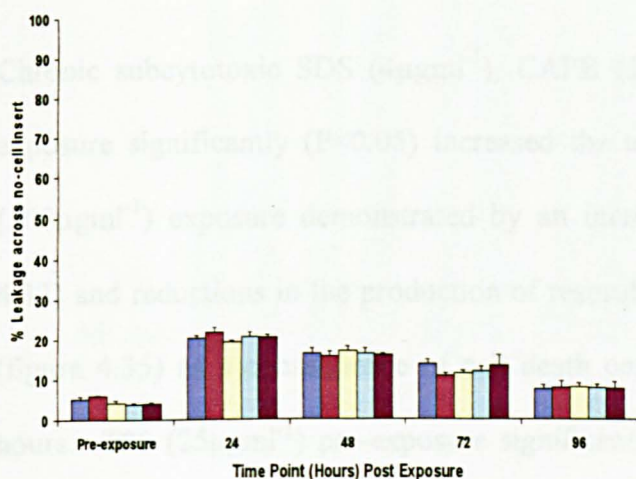
**Figure 4.27:** Leakage of fluorescein across J-HCET monolayers cultured in media containing HKGS supplements exposed to subcytotoxic concentrations of surfactants ■ control culture, ■ sodium dodecyl sulphate, ■ tween 20, ■ cocamidopropylbetaine or ■ benzalkonium chloride for 21 days and subsequently exposed (60 seconds) to an acute concentration of sodium dodecyl sulphate ( $0.8\text{mgml}^{-1}$ ), where \* denotes a significant ( $P<0.05$ ) increase and \* a decrease in fluorescein leakage compared to control at the same time point. (mean $\pm$ S.D.,  $n=3$ ).



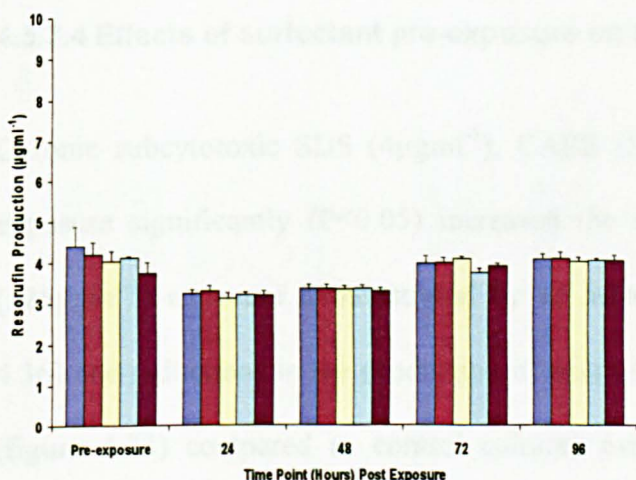
**Figure 4.28:** Resorufin production ( $\mu\text{gml}^{-1}$ ) of J-HCET monolayers cultured in media containing HKGS supplements exposed to subcytotoxic concentrations of surfactants ■ control culture, ■ sodium dodecyl sulphate, ■ tween 20, ■ cocamidopropylbetaine or ■ benzalkonium chloride for 21 days and subsequently exposed (60 seconds) to an acute concentration of sodium dodecyl sulphate ( $0.8\text{mgml}^{-1}$ ), where \* denotes a significant ( $P<0.05$ ) increase and \* a decrease in resorufin production compared to control at the same time point. (mean $\pm$ S.D.  $n=3$ ).



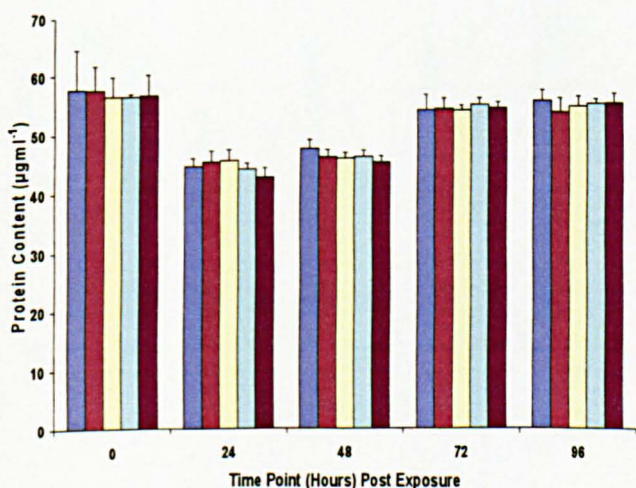
**Figure 4.29:** Protein content ( $\mu\text{gml}^{-1}$ ) of J-HCET monolayers cultured in media containing HKGS supplements exposed to subcytotoxic concentrations of surfactants ■ control culture, ■ sodium dodecyl sulphate, ■ tween 20, ■ cocamidopropylbetaine or ■ benzalkonium chloride for 21 days and subsequently exposed (60 seconds) to an acute concentration of sodium dodecyl sulphate ( $0.8\text{mgml}^{-1}$ ), where \* denotes a significant ( $P<0.05$ ) increase and \* a decrease in protein content compared to control at the same time point. (mean $\pm$ S.D.,  $n=3$ ).



**Figure 4.30:** Leakage of fluorescein across J-HCET monolayers cultured in media containing HKGS supplements exposed to subcytotoxic concentrations of surfactants ■ control culture, ■ sodium dodecyl sulphate, ■ tween 20, ■ cocamidopropylbetaine or ■ benzalkonium chloride for 21 days and subsequently exposed (60 seconds) to an acute concentration of tween 20 (300mgml<sup>-1</sup>). (mean±S.D., n=3).



**Figure 4.31:** Resorufin production (µgml<sup>-1</sup>) of J-HCET monolayers cultured in media containing HKGS supplements exposed to subcytotoxic concentrations of surfactants ■ control culture, ■ sodium dodecyl sulphate, ■ tween 20, ■ cocamidopropylbetaine or ■ benzalkonium chloride for 21 days and subsequently exposed (60 seconds) to an acute concentration of tween 20 (300mgml<sup>-1</sup>). (mean±S.D., n=3).



**Figure 4.32:** Protein content (µgml<sup>-1</sup>) of J-HCET monolayers cultured in media containing HKGS supplements exposed to subcytotoxic concentrations of surfactants ■ control culture, ■ sodium dodecyl sulphate, ■ tween 20, ■ cocamidopropylbetaine or ■ benzalkonium chloride for 21 days and subsequently exposed (60 seconds) to an acute concentration of tween 20 (300mgml<sup>-1</sup>). (mean±S.D., n=3).

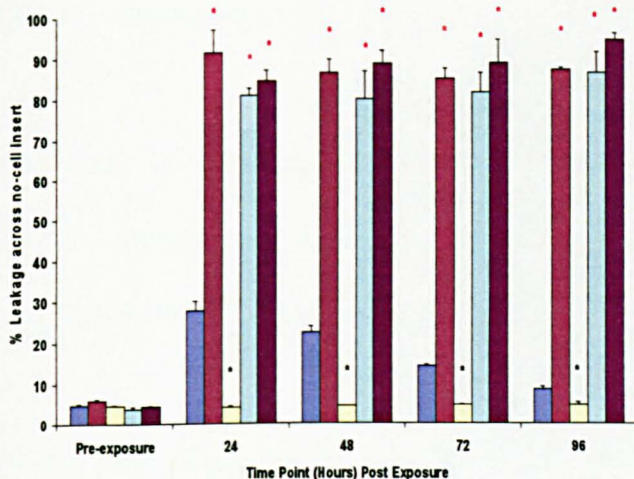
#### **4.5.3.3 Effects of surfactant pre-exposure on acute CAPB (0.5mgml<sup>-1</sup>) toxicity**

Chronic subcytotoxic SDS (4μgml<sup>-1</sup>), CAPB (3μgml<sup>-1</sup>) and BAK (0.0025μgml<sup>-1</sup>) exposure significantly (P<0.05) increased the toxicity of subsequent acute CAPB (500μgml<sup>-1</sup>) exposure demonstrated by an increase in fluorescein leakage (figure 4.33) and reductions in the production of resorufin (figure 4.34) and protein content (figure 4.35) as a consequence of cell death compared to control cultures over 96 hours. T20 (25μgml<sup>-1</sup>) pre-exposure significantly (P<0.05) reduced the toxicity of subsequent acute CAPB (500μgml<sup>-1</sup>) exposure.

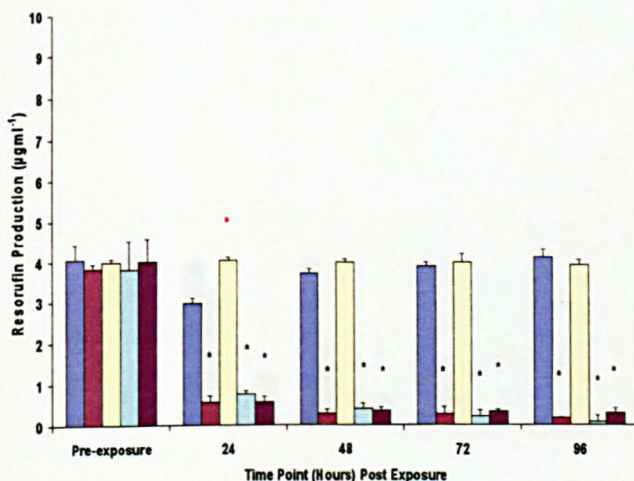
#### **4.5.3.4 Effects of surfactant pre-exposure on acute BAK (125μgml<sup>-1</sup>) toxicity**

Chronic subcytotoxic SDS (4μgml<sup>-1</sup>), CAPB (3μgml<sup>-1</sup>) and BAK (0.0025μgml<sup>-1</sup>) exposure significantly (P<0.05) increased the toxicity of subsequent acute BAK (125μgml<sup>-1</sup>) exposure demonstrated by an increase in fluorescein leakage (figure 4.36) and reductions in the production of resorufin (figure 4.37) and protein content (figure 4.38) compared to control cultures over 96 hours. T20 (25μgml<sup>-1</sup>) pre-exposure did not significantly modulate the toxicity of subsequent acute BAK (125μgml<sup>-1</sup>) exposure.

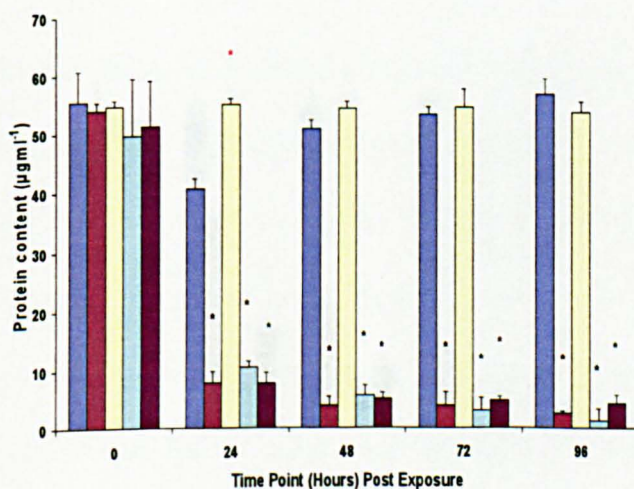




**Figure 4.33:** Leakage of fluorescein across J-HCET monolayers cultured in media containing HKGS supplements exposed to subcytotoxic concentrations of surfactants (■ control culture, ■ sodium dodecyl sulphate, ■ tween 20, ■ cocamidopropylbetaine or ■ benzalkonium chloride for 21 days and subsequently exposed (60 seconds) to an acute concentration of cocamidopropylbetaine ( $0.5\text{mgml}^{-1}$ ), where \* denotes a significant ( $P<0.05$ ) increase and \* a decrease in fluorescein leakage compared to control at the same time point. (mean $\pm$ S.D.,  $n=3$ ).

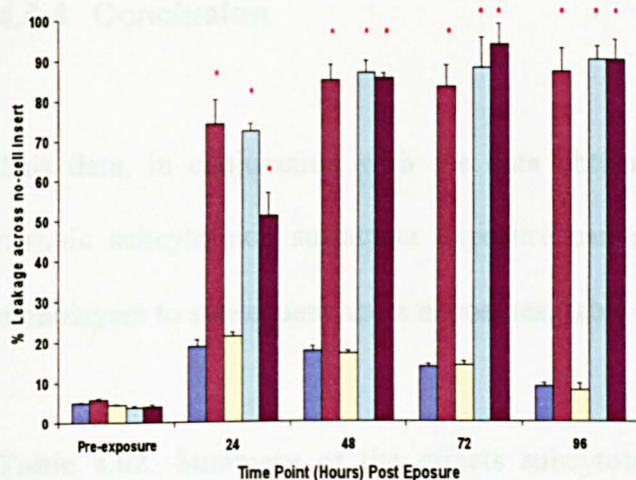


**Figure 4.34:** Resorufin production ( $\mu\text{gml}^{-1}$ ) of J-HCET monolayers cultured in media containing HKGS supplements exposed to subcytotoxic concentrations of surfactants (■ control culture, ■ sodium dodecyl sulphate, ■ tween 20, ■ cocamidopropylbetaine or ■ benzalkonium chloride for 21 days and subsequently exposed (60 seconds) to an acute concentration of cocamidopropylbetaine ( $0.5\text{mgml}^{-1}$ ), where \* denotes a significant ( $P<0.05$ ) increase and \* a decrease in resorufin production compared to control at the same time point. (mean $\pm$ S.D.,  $n=3$ ).

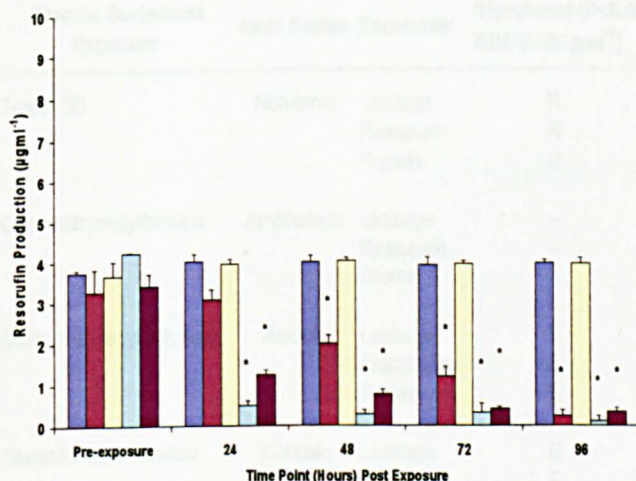


**Figure 4.35:** Protein content ( $\mu\text{gml}^{-1}$ ) of J-HCET monolayers cultured in media containing HKGS supplements exposed to subcytotoxic concentrations of surfactants (■ control culture, ■ sodium dodecyl sulphate, ■ tween 20, ■ cocamidopropylbetaine or ■ benzalkonium chloride for 21 days and subsequently exposed (60 seconds) to an acute concentration of cocamidopropylbetaine ( $0.5\text{mgml}^{-1}$ ), where \* denotes a significant ( $P<0.05$ ) increase and \* a decrease in protein content compared to control at the same time point. (mean $\pm$ S.D.,  $n=3$ ).

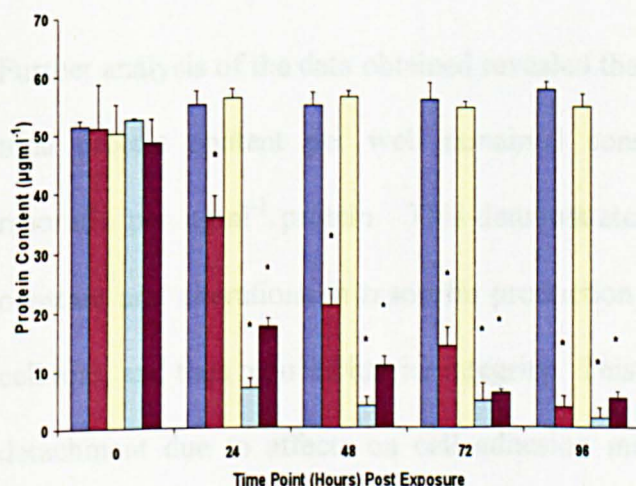




**Figure 4.36:** Leakage of fluorescein across J-HCET monolayers cultured in media containing HKGS supplements exposed to subcytotoxic concentrations of surfactants ■ control culture, ■ sodium dodecyl sulphate, ■ tween 20, ■ cocamidopropylbetaine or ■ benzalkonium chloride for 21 days and subsequently exposed (60 seconds) to an acute concentration of benzalkonium chloride ( $125\mu\text{gml}^{-1}$ ), where \* denotes a significant ( $P<0.05$ ) increase in fluorescein leakage compared to control at the same time point. (mean $\pm$ S.D.,  $n=3$ ).



**Figure 4.37:** Resorufin production ( $\mu\text{gml}^{-1}$ ) of J-HCET monolayers cultured in media containing HKGS supplements exposed to subcytotoxic concentrations of surfactants ■ control culture, ■ sodium dodecyl sulphate, ■ tween 20, ■ cocamidopropylbetaine or ■ benzalkonium chloride for 21 days and subsequently exposed (60 seconds) to an acute concentration of benzalkonium chloride ( $125\mu\text{gml}^{-1}$ ), where \* denotes a significant ( $P<0.05$ ) decrease in resorufin production compared to control at the same time point. (mean $\pm$ S.D.,  $n=3$ ).



**Figure 4.38:** Protein content ( $\mu\text{gml}^{-1}$ ) of J-HCET monolayers cultured in media containing HKGS supplements exposed to subcytotoxic concentrations of surfactants ■ control culture, ■ sodium dodecyl sulphate, ■ tween 20, ■ cocamidopropylbetaine or ■ benzalkonium chloride for 21 days and subsequently exposed (60 seconds) to an acute concentration of benzalkonium chloride ( $125\mu\text{gml}^{-1}$ ), where \* denotes a significant ( $P<0.05$ ) decrease in protein content compared to control at the same time point. (mean $\pm$ S.D.,  $n=3$ ).

4.5.4 Conclusion

This data, in conjunction with the data obtained in chapter 3 demonstrates that chronic subcytotoxic surfactant exposure can modulate the response of J-HCET monolayers to subsequent acute exposures (table 4.02).

**Table 4.02:** Summary of the effects subcytotoxic surfactant exposure has on a subsequent acute exposure.

Chronic Surfactant Exposure	Ionic Status	Parameter	Significant (P<0.05) change in toxicity of			
			SDS (0.8mgml <sup>-1</sup> )	T20 (300mgml <sup>-1</sup> )	CAPB (0.5mgml <sup>-1</sup> )	BAK (125µgml <sup>-1</sup> )
Tween 20	Non-ionic	Leakage	R	-	R	-
		Resazurin	R	-	R	-
		Protein	R	-	R	-
Cocamidopropylbetaine	Amphoteric	Leakage	-	-	E	E
		Resazurin	-	-	E	E
		Protein	-	-	E	E
Sodium dodecyl sulphate	Anionic	Leakage	E	-	E	E
		Resazurin	E	-	E	E
		Protein	E	-	E	E
Benzalkonium chloride	Cationic	Leakage	E	-	E	E
		Resazurin	E	-	E	E
		Protein	E	-	E	E

Where E denotes enhanced and R denotes reduced toxicity of the acute surfactant.

Further analysis of the data obtained revealed that the ratio of resazurin production to total protein content per well remained constant at approximately 0.08µgml<sup>-1</sup> resorufin per µgml<sup>-1</sup> protein. This demonstrated that the culture activity remained constant and alterations in resorufin production or fluorescein leakage were due to cell loss, and thus reduced barrier integrity. This loss was also likely to be due to cell detachment due to affects on cell adhesion molecules rather than necrosis, since resazurin per protein was unaffected. Hence a more detailed investigation of the adhesion molecule expression and location was undertaken.



## **Chapter 4b**

***Effects of medium composition on barrier function***

**“Morphology and adhesion molecule expression”**

# Effects of medium composition on barrier function

## “Morphology and adhesion molecule expression”

### 4.6 Modulation of J-HCET monolayer morphology by growth media calcium concentration

#### 4.6.1 Introduction

The corneal epithelium functions as a barrier that isolates the eye from the outside environment (Ban *et al.*, 2003). For *in vitro* models to mimic this function a confluent sheet of cells maintaining the distinct internal environment from the extra insert well compartment the paracellular pathway between adjacent cells in the sheet must be tightly sealed to prevent the diffusion of solutes, whilst the apical and basolateral domains must be differentiated to allow active transport across the sheet (Tsukita and Furuse, 1999). Tight junctions (TJ) are directly involved in paracellular sealing (barrier function) and *in vivo* provide a continuous seal around the apical aspect of adjoining epithelial cells, thereby, preventing the free passage of molecules between adjacent epithelial cells (paracellular pathway). Thus for the *in vitro* model to be representative the cells should not only express the appropriate adhesion molecules but do so in the relevant locations. TJ complex includes two integral transmembrane proteins: claudin; occludin and membrane associated proteins such as zonula occludins-1 (ZO-1), ZO-2 and ZO-3 (Tsukita *et al.*, 2001). ZO-1 has a molecular mass of 220kD (Stevenson *et al.*, 1986; Lapierre 2000) was one of the first proteins identified and localized to the TJ where in conjunction with ZO-2, and ZO-3 (Lapierre 2000) acts as a scaffolding or plaque protein responsible for connecting the

proteins occludin and the claudins to the f-actin cytoskeleton (Citi, 1993; Anderson, 2001; Poritz *et al.*, 2004). There are antibodies available to these adhesion molecules so that their location *in vitro* and *in vivo* can be determined. Sugrue and Zieske (1997) showed that the expression and localization to the interior surface of the plasma membrane of ZO-1. It now appears that ZO-1 is a ubiquitous component of the tight junction in all epithelia and endothelia (Sugrue and Zieske, 1996) interacting with occludin (Stevenson *et al.*, 1986; Citi, 1993; Tsukita and Furuse, 1999) an integral membrane protein involved in the permeability barrier function of the tight junction. However, it has been shown that ZO-1 may be found at non-tight junctional locations (Schnabel *et al.*, 1990; Howarth *et al.*, 1992; Miragall *et al.*, 1994) leading to the unfortunate misinterpretation that the presence of ZO-1 immunoreactivity equates to the presence of tight junctions (Sugrue and Zieske, 1996). Occludin is a 60 – 65kDa protein with four transmembrane domains (Furuse *et al.*, 1993; Balda and Matter 2000; Ban *et al.*, 2003) with both N- and C- termini oriented into the cytoplasm (Balda and Matter 2000). Occludin appears to be the most important and relatively well characterized transmembrane protein of the tight junction (Kale *et al.*, 2003) with the C-terminal interacting *in vitro* with several intracellular scaffolding proteins including ZO-1, ZO-2, ZO-3 (Furuse *et al.*, 1993; Balda and Matter 2000; Ban *et al.*, 2003) and f-actin (Goodenough, 1999; Wittchen *et al.*, 1999 ). To date there have been no reports of occludin isoforms – that is, different occludin-related genes – although two forms of occludin are generated by alternate splicing (Morita *et al.*, 1999; Tsukita and Furuse, 1999). The level of occludin expression in various cell types correlates well with the number of tight junction strands (Saitou *et al.*, 1997; Tsukita and Furuse, 1999). Claudins, 23kDa integral membrane proteins bearing four transmembrane domains, have been

identified as components of TJ strands (Furuse *et al.*, 1998; Tsukita and Furuse 2002), and to be directly involved in intercellular sealing in simple epithelia (Anderson 2001; Tsukita *et al.*, 2001; Balda and Matter, 2000; Furuse *et al.*, 2002; Ban *et al.*, 2003). Claudin-1 and -2 were originally identified because they cofractionated with occludin from sonicated fractions (Furuse *et al.*, 1998). There is no evidence for a distinct interaction between occludin and claudin (Balda and Matter 2000), and claudins do not copurify with occludin from detergent solubilised membranes (Balda and Matter 2000). At least 24 members of the claudin family have been identified (Furuse *et al.*, 1998; Gow *et al.*, 1999; Tsukita and Furuse, 2002). In contrast to occludin, individual claudins are generally expressed in only a restricted number of specific cell types, suggesting that they are associated with tissue-specific functions of tight junctions (Balda and Matter 2000) but the level of claudin expression does not correlate with the number of tight junction strands (Saitou *et al.*, 1997; Tsukita and Furuse, 1999). Besides their membrane topology, different claudin members share other structural features, suggested by the observation that eight different claudins can bind to the three submembrane proteins ZO-1, ZO-2 and ZO-3 by interaction involving the claudins C-terminal cytosolic domain and the PDZ domain of the submembrane plaque proteins (Simon, 1999). Whilst the cells do not appear to have their activity and cell number modulated by transfer into variable calcium concentrations over 72 hours, it is important to confirm that subtle or significant changes were not occurring morphologically or in the nature of key adhesion molecules, responsible for barrier integrity. Initially the effects of calcium concentrations were examined.

#### **4.6.2 Materials and Methods**

J-HCET were resuspended in growth media containing 0, 60, 80, 100, 500 or 1000 $\mu$ M calcium chloride and seeded at  $3 \times 10^5$  cells ml<sup>-1</sup> (200 $\mu$ l) into 8 well chamber slides. Cells were incubated at 37°C, 5% CO<sub>2</sub> (v/v) in air for 72 hours. Slides were examined using a Nikon phase contrast/fluorescence microscope.

#### **4.6.3 Results**

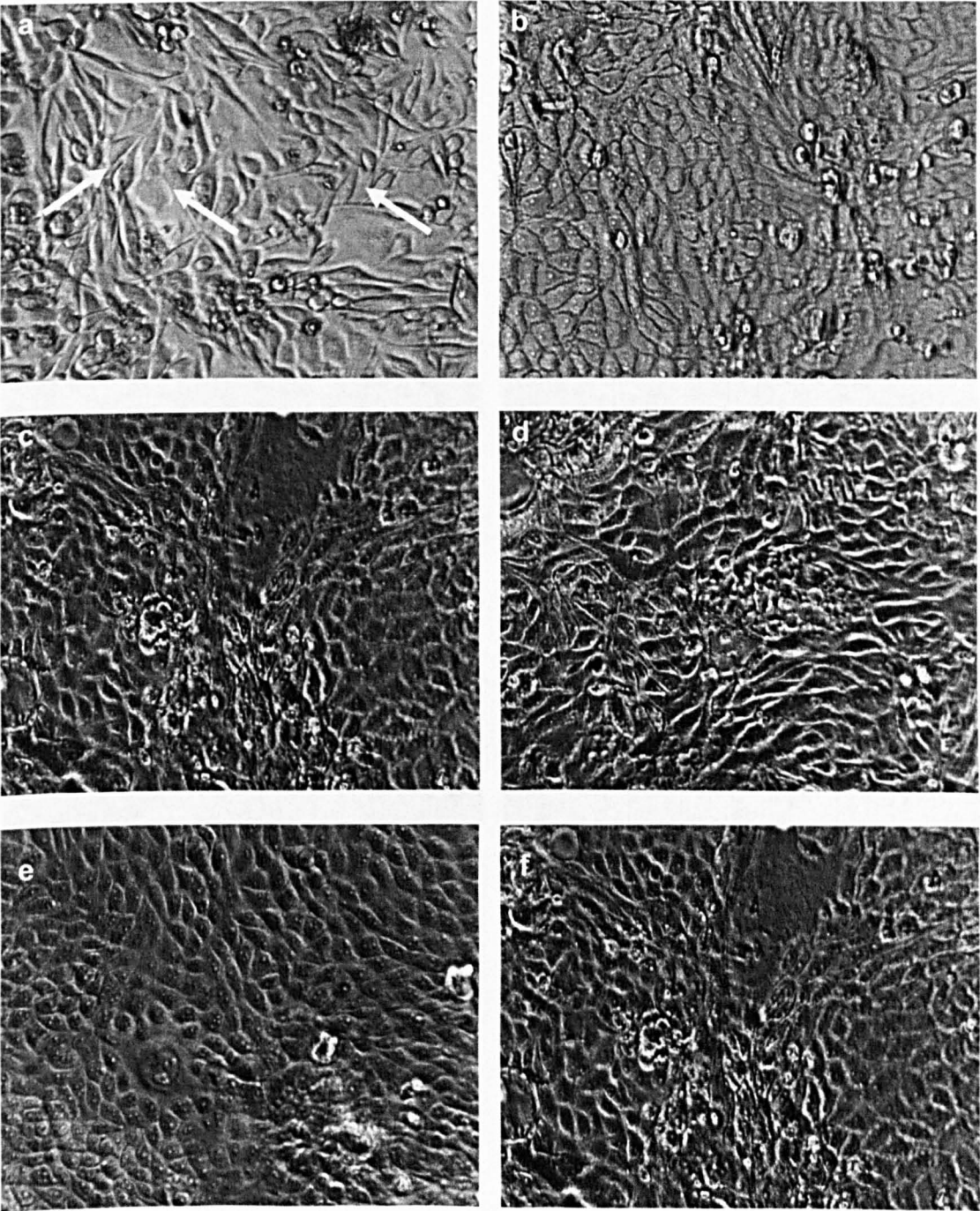
Despite the omission of calcium from the growth media, J-HCET retained the ability to proliferate but the monolayers revealed individual J-HCET clearly distinguishable from neighbouring cells (figure 4.39a and 4.40a). At calcium concentration of 60 $\mu$ M and above, J-HCET monolayers cultured in either HKGS (figure 4.39b/c/d/e/f) or HKGS-V2 (figure 4.40b/c/d/e/f) supplemented media adopt the typical “cobblestone like” appearance commonly described in studies (Furuse *et al.*, 1993; Balda and Matter 2000; Ban *et al.*, 2003) utilising corneal cell lines, with no visible gaps between the cells.

#### **4.6.4 Conclusions**

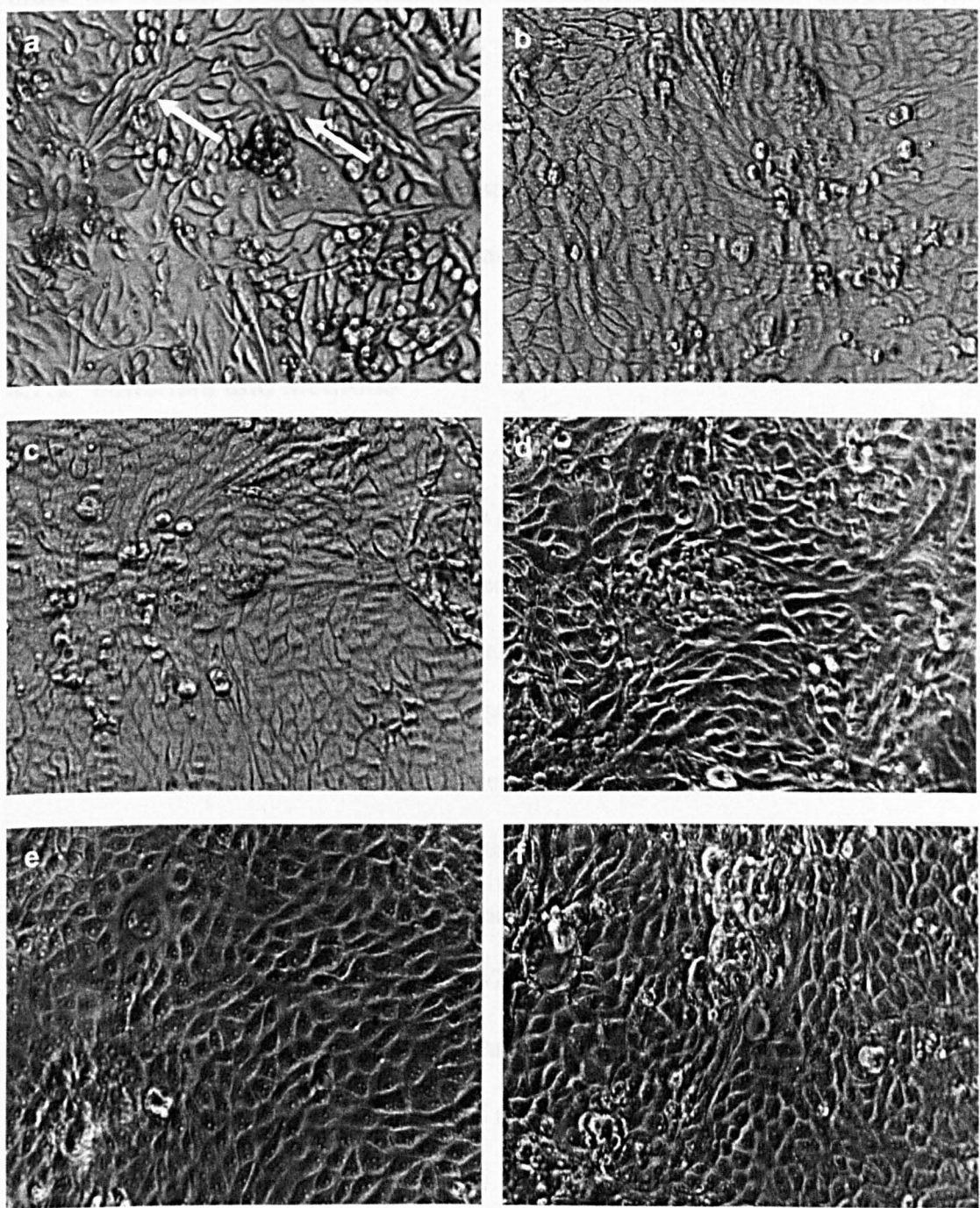
The corneal cells can grow for up to 72 hours in the absence of exogenous medium calcium but reveal compromised morphology and cell-cell contact. The differing growth supplements do not appear to affect these responses. Thus the endogenous calcium reserves, from growth routinely in 60 $\mu$ M calcium containing Epilife™ medium, allow the cells to grow in the normal fashion, but appears to affect cell

adhesion. This affect on adhesion could either be due to reduced adhesion molecule/s levels or mislocation.

**Figure 4.39a/b/c/d/e/f:** Greyscale phase contrast micrographs of J-HCET monolayers cultured in HKGS supplemented Epilife media containing a) 0 b) 60 c) 80 d) 100 e) 500 and f) 1000 $\mu$ M calcium. (x400 magnification).



**Figure 4.40a/b/c/d/e/f:** Greyscale phase contrast micrographs of J-HCET monolayers cultured in HKGS-V2 supplemented Epilife media containing a) 0 b) 6 c) 80 d) 100 e) 500 and f) 1000 $\mu$ M calcium. (x400 magnification).





## **4.7 Modulation of f-actin by growth media calcium concentration**

### **4.7.1 Introduction**

Since the transfer to 0 $\mu$ M calcium medium results in a modified morphological appearance and since 60 $\mu$ M and 80 $\mu$ M affect adversely the barrier function, as measured by sodium fluorescein leakage (reported in chapter 4a), but morphology was normal, the cytoskeleton was investigated. Fibrous actin (f-actin) plays an important role in anchorage of key adhesion molecules.

### **4.7.2 Materials and Methods**

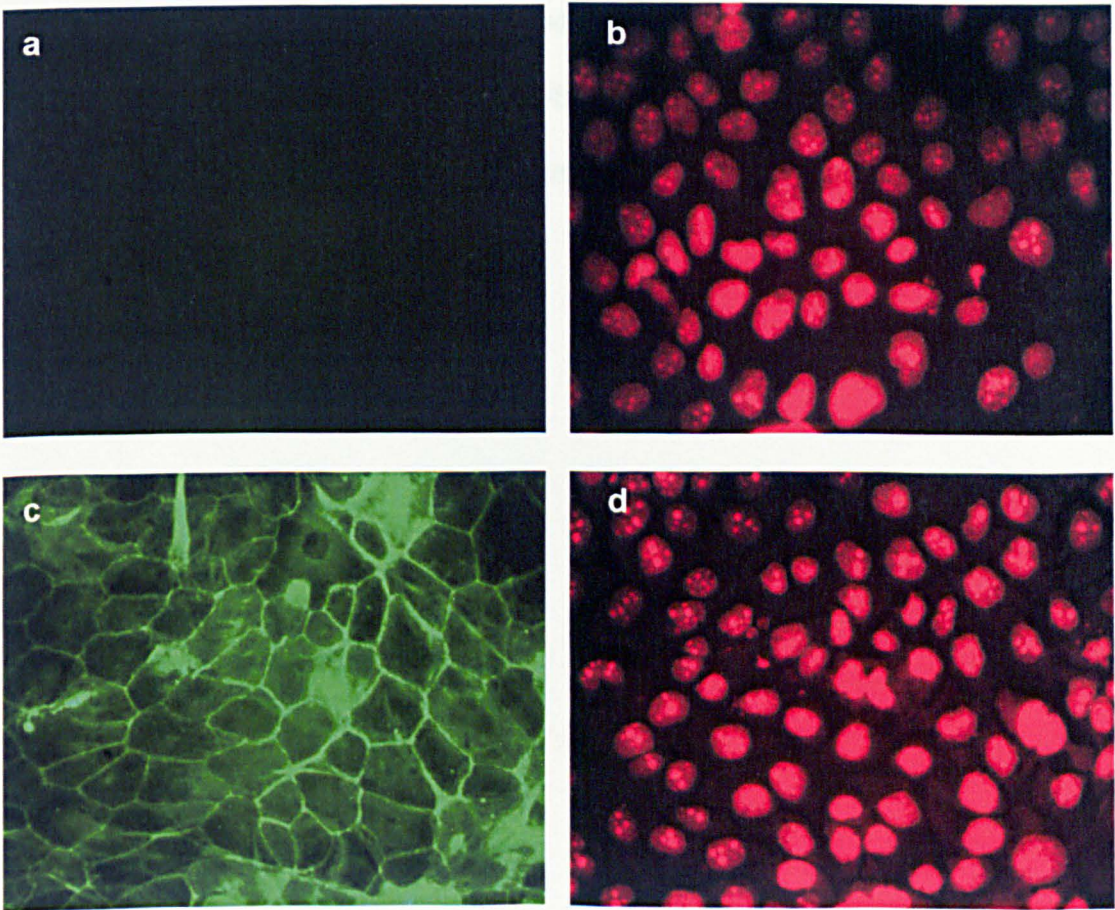
J-HCET were seeded at the same density and calcium concentrations as for section 4.2 into 8 well chamber slides. Cells were incubated at 37°C, 5% CO<sub>2</sub> (v/v) in air for 72 hours. The cultures were fixed in paraformaldehyde solution (1% w/v in PBS) for 30 minutes. Triton X-100 (200 $\mu$ l; 0.1% v/v in PBS) was added for 5 minutes to permeabilize the cells. Monolayers were rinsed with BSA solution (0.1% w/v in PBS) and washed in fresh BSA solution. Fluorescein isothiocyanate (FITC) conjugated phalloidin (1mgml<sup>-1</sup>) was suspended in 1.5ml PBS and 200 $\mu$ l applied to the chambers. Slides were incubated at 37°C, 5% CO<sub>2</sub> (v/v) in air for 1 hour in the dark. Slides were rinsed with BSA solution and washed in fresh BSA solution. Slides were washed in water and mounted using Vectashield™ containing propidium iodine (50 $\mu$ gml<sup>-1</sup>). Immunostaining of J-HCET monolayers cultured in the keratinocyte media (KGM) supplemented with animal derived products and calcium (100 $\mu$ M) routinely used in the ICCVAM/ECVAM validation trial (Strickland *et al.*,

2003) was employed as a control (figure 4.41). Slides were viewed under a Leica Confocal Microscope.

### 4.7.3 Results

In control J-HCET monolayers the absence of phalloidin staining (figure 4.41a) demonstrates no auto-fluorescence at the wavelengths used to visualise the phalloidin binding.

**Figure 4.41a/b/c/d:** (a) KGM cultured J-HCET monolayer PBS alone (b) the same J-HCET monolayer stained with propidium iodide nuclear staining. (c) KGM cultured J-HCET monolayer with FITC conjugated phalloidin (d) the same J-HCET monolayer with propidium iodide staining of the nuclei. (x400 magnification)



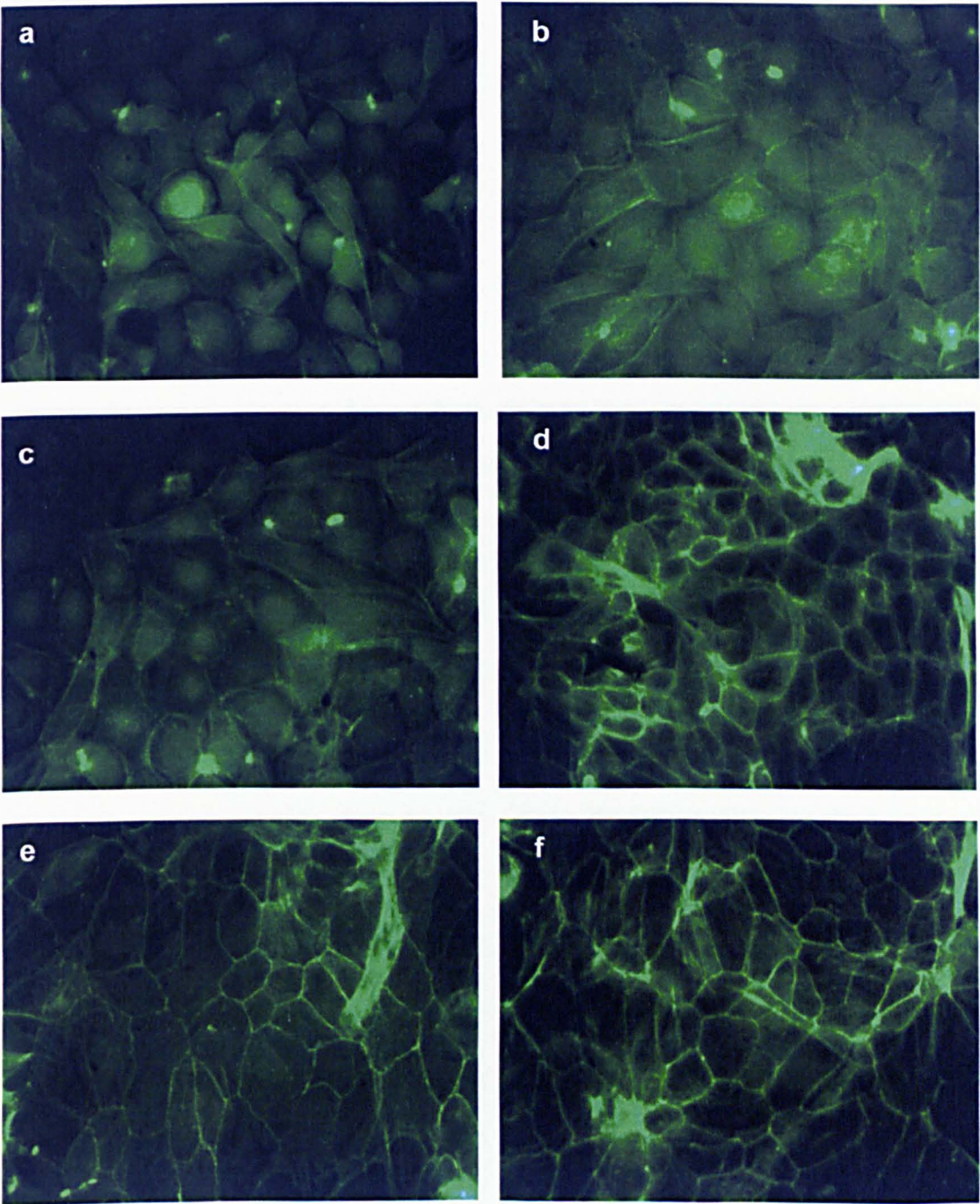
The staining with propidium iodide confirmed the presence of a complete monolayer which had been active prior to fixation since distinct nucleoli can be observed (figure 4.41b and 4.41d). The combined staining confirmed no cross fluorescence signal between phalloidin and propidium iodide (figure 4.41c/d). When J-HCET were culture in medium containing HKGS supplements to obtain complete localisation of the phalloidin binding to the periphery of the cells at least 500 $\mu$ M calcium in the medium was required (figure 4.42e/f). Marked membrane association was also observed at 100 $\mu$ M calcium (figure 4.42d). HKGS-V2 supplements produced a similar pattern except that the 100 $\mu$ M calcium did not induce the same degree of membrane localisation (figure 4.43d and figure 4.42d).

#### **4.7.4 Conclusions**

A calcium concentration of at least 500 $\mu$ M is required for the expression and correct intracellular localisation of f-actin in J-HCET cells cultured in either HKGS or HKGS-V2 supplemented Epilife™ medium.

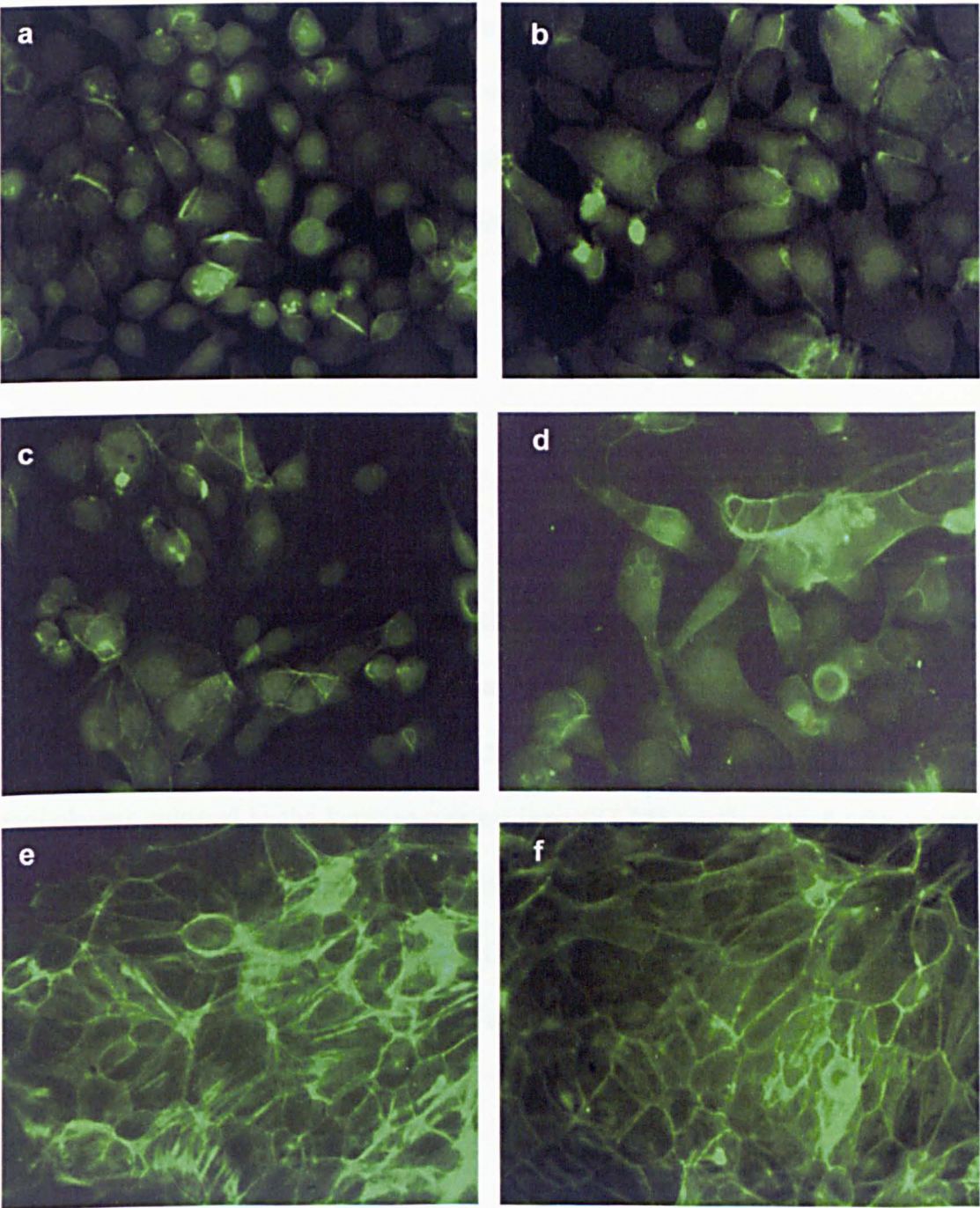


**Figure 4.42a/b/c/d/e/f:** Fluorescence micrograph of J-HCET cell FITC-phalloidin stained for f-actin. HKGS supplemented Epilife media containing a) 0 b) 60 c) 80 d) 100 e) 500 and f) 1000 $\mu$ M calcium. (x400 magnification).





**Figure 4.43a/b/c/d/e/f:** Fluorescence micrograph of J-HCET cell FITC-phalloidin stained for f-actin. HKGS-V2 supplemented Epilife media containing a) 0 b) 60 c) 80 d) 100 e) 500 and f) 1000 $\mu$ M calcium. (x400 magnification).



## **4.8 Modulation of ZO-1 by growth media calcium concentration**

### **4.8.1 Introduction**

Having established that calcium concentrations affect f-actin distribution the effects on adhesion molecules in the two media was evaluated. Studies were conducted to determine the effects of growth medium calcium concentration have upon the expression and localisation of ZO-1 in J-HCET monolayers, since ZO-1 is located in TJ and other adhesion junctions.

### **4.8.2 Materials and Methods**

J-HCET seeded at the same density and calcium concentrations as for section 4.2 into 8 well chamber slides. Cells were incubated at 37°C, 5% CO<sub>2</sub> (v/v) in air for 72 hours. Indirect immunohistochemistry was performed as outlined in section 2.2.5.1 using a mouse anti-ZO-1 primary antibody (table 2.0). Immunostaining of J-HCET monolayers cultured in the keratinocyte medium (KGM) supplemented with animal derived products and calcium to give a final concentration of 100µM routinely used in the ICCVAM/ECVAM validation trial (Strickland *et al.*, 2003) was employed as a control (figure 4.44). Slides were viewed under a Leica Confocal Microscope.

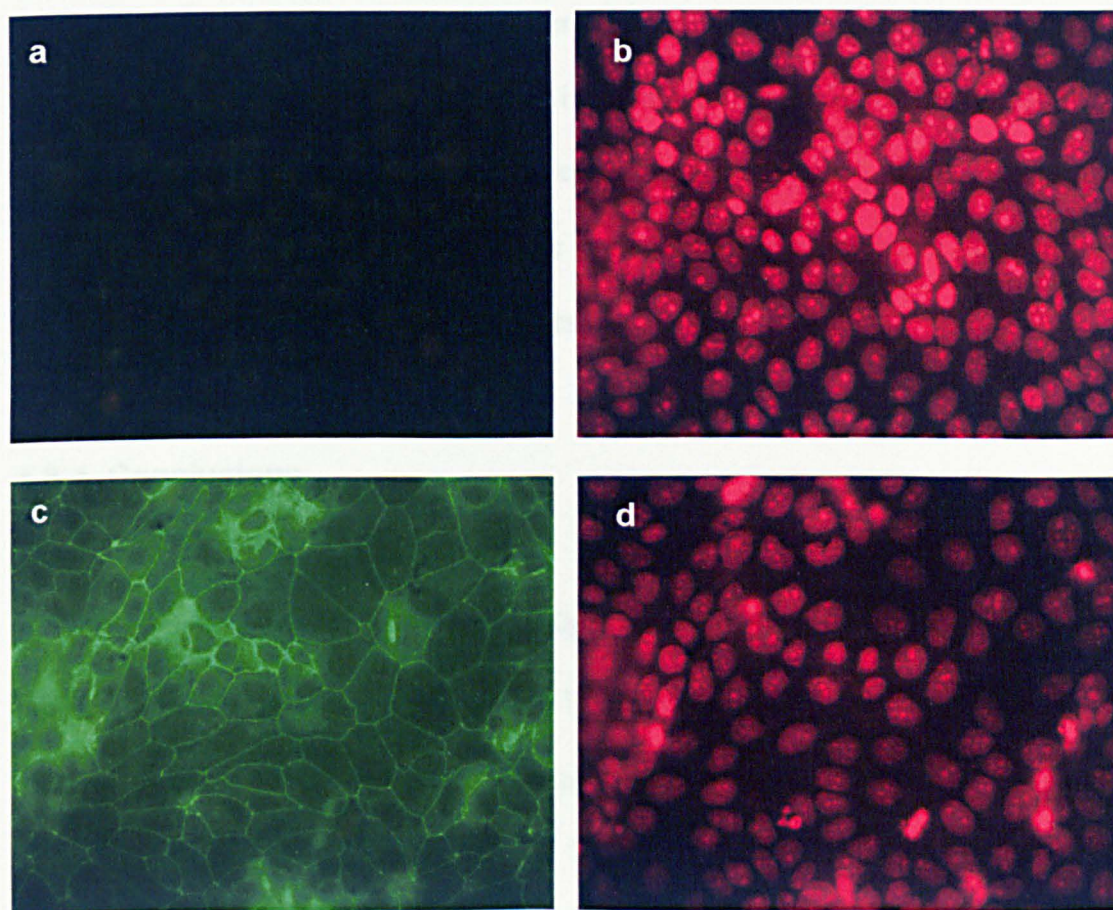
### **4.8.3 Results**

In control J-HCET monolayers the absence of ZO-1 immunostaining (figure 4.44a) demonstrates no auto-fluorescence at the wavelengths used to visualise the FITC



labelled anti-ZO-1 binding. The staining with propidium iodide confirmed the presence of a complete active monolayer (figure 4.44b). The combined staining confirmed no cross fluorescence signal between ZO-1 and propidium iodide (figure 4.44c/d).

**Figure 4.44a/b/c/d:** (a) KGM cultured J-HCET monolayer in PBS no primary antibody but exposed to secondary antibody. (b) The same J-HCET monolayer propidium iodide staining. (c) KGM cultured J-HCET monolayer binding of mouse anti-ZO-1. (d) The same J-HCET monolayer propidium iodide staining. (x400 magnification).





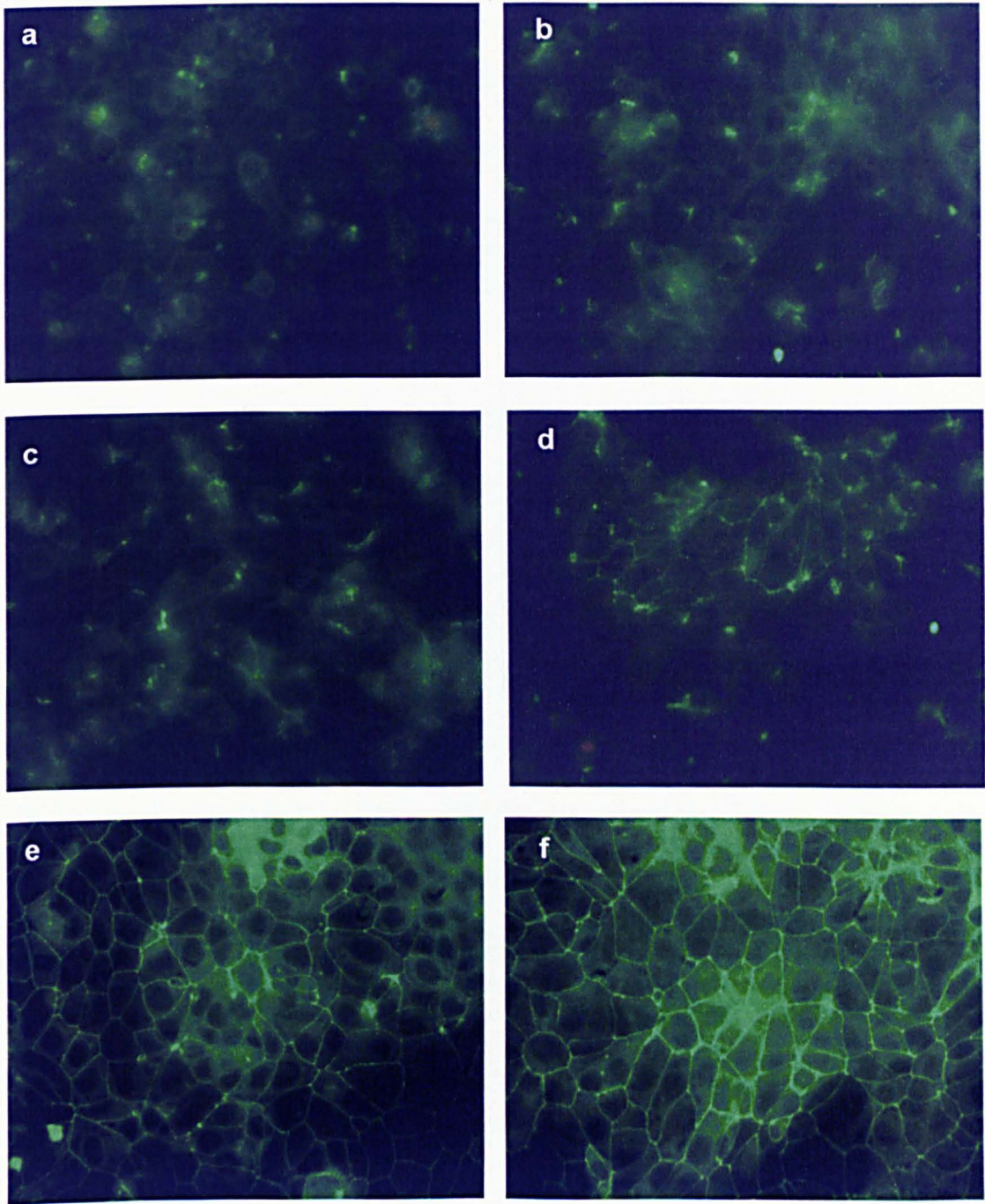
When J-HCET were culture in medium containing HKGS supplements, at low calcium concentrations (0 $\mu$ M to 80 $\mu$ M) ZO-1 expression was not membrane localised but diffuse within the cytoplasm (figure 4.45a/b/c). When J-HCET were cultured in media containing 100 $\mu$ M calcium, there was limited localization to the plasma membrane but most immunostaining remains diffuse within the cytoplasm (figure 4.45d). At medium concentrations of calcium at 500 $\mu$ M and 1000 $\mu$ M, ZO-1 immunostaining completely encircles the squamous epithelial cells (figure 4.45e/f). ZO-1 staining appears as clusters of immunoreactivity especially at contact points between three or more cells; this may reflect overlapping of membrane contacts. When J-HCET were cultured in medium containing HKGS-V2 supplements, at low calcium concentrations (0 $\mu$ M to 100 $\mu$ M) ZO-1 was present but diffuse within the cytoplasm and not located to the plasma membrane (figure 4.46a/b/c/d). At calcium concentrations of 500 $\mu$ M and 1000 $\mu$ M in complete media ZO-1 immunostaining completely encircles the large squamous epithelial cells (figure 4.46e/f). It was also evident that ZO-1 staining appears in the same pattern as observed in the Epilife™ supplemented with the HKGS supplements.

#### **4.8.4 Conclusions**

A calcium concentration of at least 500 $\mu$ M was required for the expression and correct intracellular localisation of ZO-1 to the cell membrane in J-HCET cells cultured in either HKGS or HKGS-V2 supplemented Epilife™ medium. The pattern of ZO-1 expression was suggestive of being compatible with a competent seal between adjacent abutting cells, indicative of the presence of a functional barrier to paracellular flow, regardless of the medium growth factor supplementation. Thus

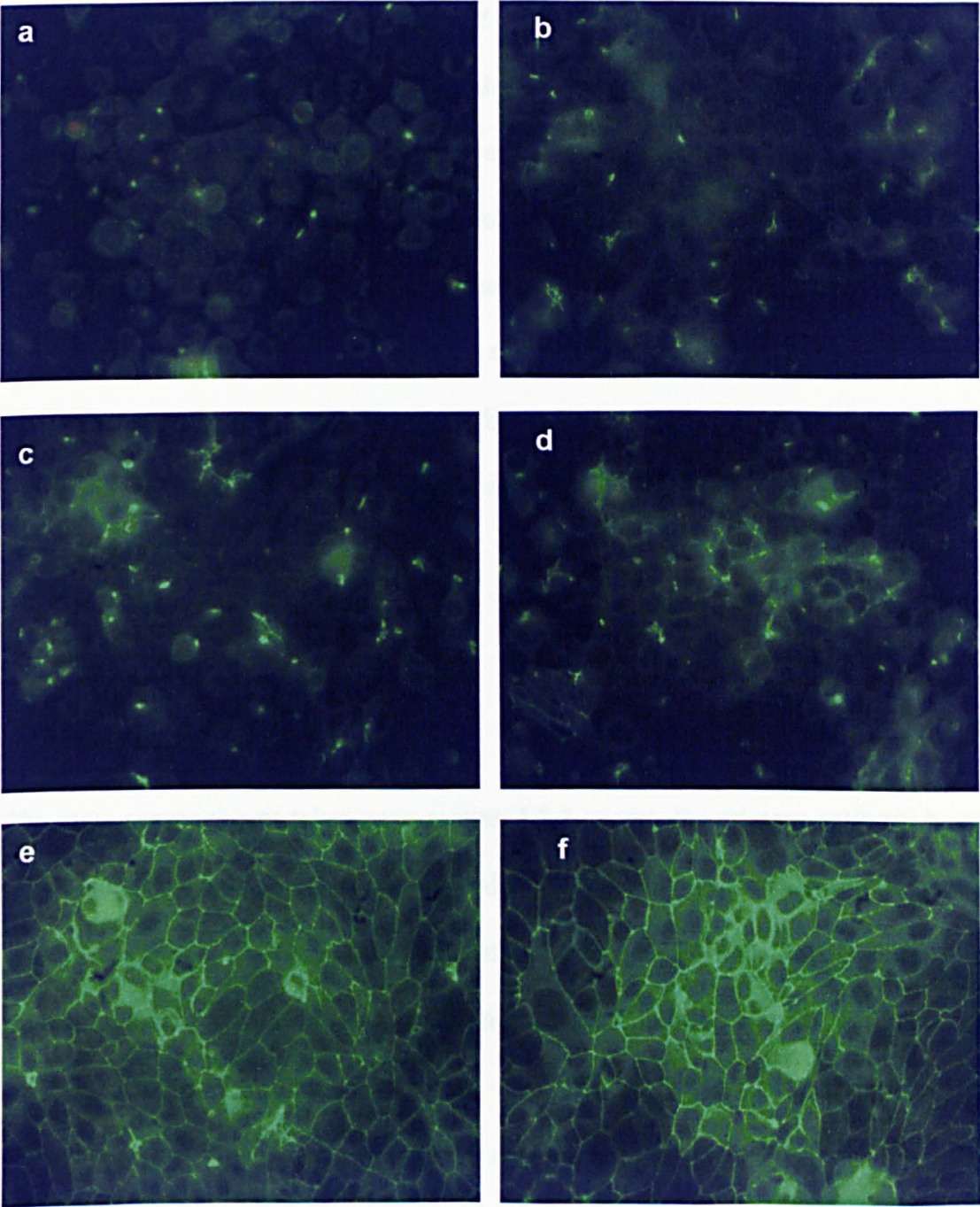
from the FL data it would appear that ZO-1 is associated with the TJ's, and that HKGS-V2 does not inhibit TJ formation.

**Figure 4.45a/b/c/d/e/f:** Immunofluorescent staining of ZO-1 in J-HCET monolayer cultured in HKGS supplemented Epilife media containing a) 0 b) 60 c) 80 d) 100 e) 500 and f) 1000 $\mu$ M calcium. (x400 magnification).





**Figure 4.46a/b/c/d/e/f:** Immunofluorescent staining of ZO-1 in J-HCET monolayer cultured in HKGS-V2 supplemented Epilife media containing a) 0 b) 60 c) 80 d) 100 e) 500 and f) 1000 $\mu$ M calcium. (x400 magnification).



## **4.9 Modulation of occludin by growth media calcium concentration**

### **4.9.1 Introduction**

Since ZO-1 is ubiquitous (Schnabel *et al.*, 1990; Howarth *et al.*, 1992; Miragall *et al.*, 1994) to establish the morphological correlation between barrier function and tight junctions another adhesion molecule was examined. Despite the ZO-1 being localised, it is an internal membrane marker, not a transmembrane component of the tight junction. Occludin was the first transmembrane protein of tight junctions that was identified (Furuse *et al.*, 1993; Balda and Matter 2000). Studies were conducted to determine the effects of growth medium calcium concentration has upon the expression and localisation of occludin in J-HCET monolayers, to confirm effects upon tight junctions.

### **4.9.2 Materials and Methods**

The same approach was taken as in section 4.8.2 except; the primary antibody being rabbit anti-occludin (table 2.0) was diluted in 0.1% BSA (w/v) in PBS and incubated with the cells at 4<sup>o</sup>C overnight. Immunostaining of J-HCET monolayers cultured in the keratinocyte media (KGM) supplemented with animal derived products and calcium (100µM) routinely used in the ICCVAM/ECVAM validation trial (Strickland *et al.*, 2003) was employed as a control (figure 4.47). Slides were viewed under a Leica Confocal Microscope.

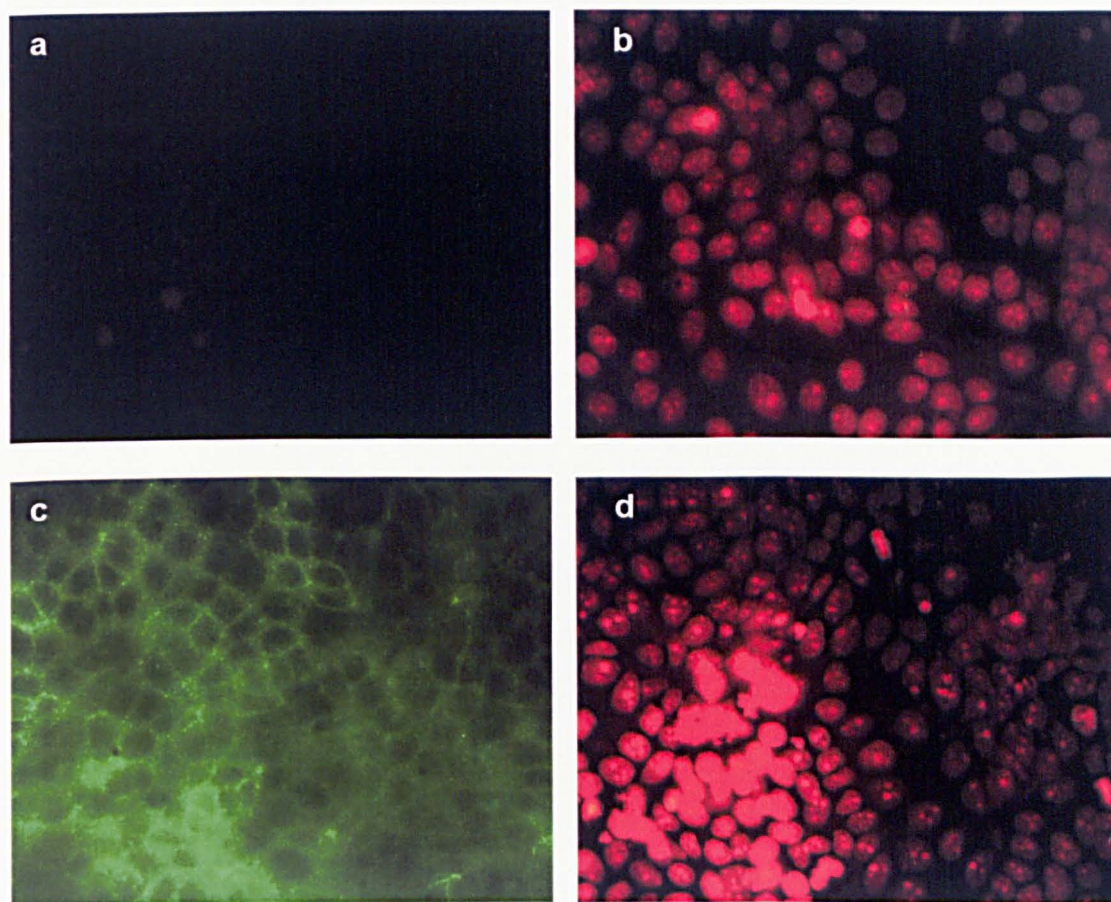
### 4.9.3 Results

In control J-HCET monolayers the absence of occludin immunostaining (figure 4.47a) demonstrates no auto-fluorescence at the wavelengths used to visualise the FITC labelled anti-occludin binding. The staining with propidium iodide confirmed the presence of a complete active monolayer (figure 4.47b). The combined staining confirmed no cross fluorescence signal between occludin and propidium iodide (figure 4.47c/d). Staining of the KGM cultured controls also confirms a scattered pattern of occludin distribution at the margins of the cells that form the monolayers.

With this more diffuse pattern of staining, it was more difficult to observe variations in the distribution of the staining pattern. It does appear that at 100 $\mu$ M and above (figure 4.48d/e/f) the, distribution and concentration hot spots were very similar to control staining. At 80 $\mu$ M there appears to be fainter staining, but still located to the margins of the cells (figure 4.48c). There was a marked contrast with the HKGS-V2 supplements, with more concentrated localisation to the membranes but only at 500 $\mu$ M calcium and above (figure 4.49a/b/c/d versus e/f). As a result of the more diffuse staining pattern it was difficult to determine if the level of staining was altered or just its localisation.



**Figure 4.47a/b/c/d:** (a) KGM cultured J-HCET monolayer exposed to PBS. (b) J-HCET monolayer stained with propidium iodide. (c) KGM cultured J-HCET monolayer exposed to primary mouse anti-occludin then secondary FITC antibody. (d) Same J-HCET monolayer propidium iodide staining. (x400 magnification).



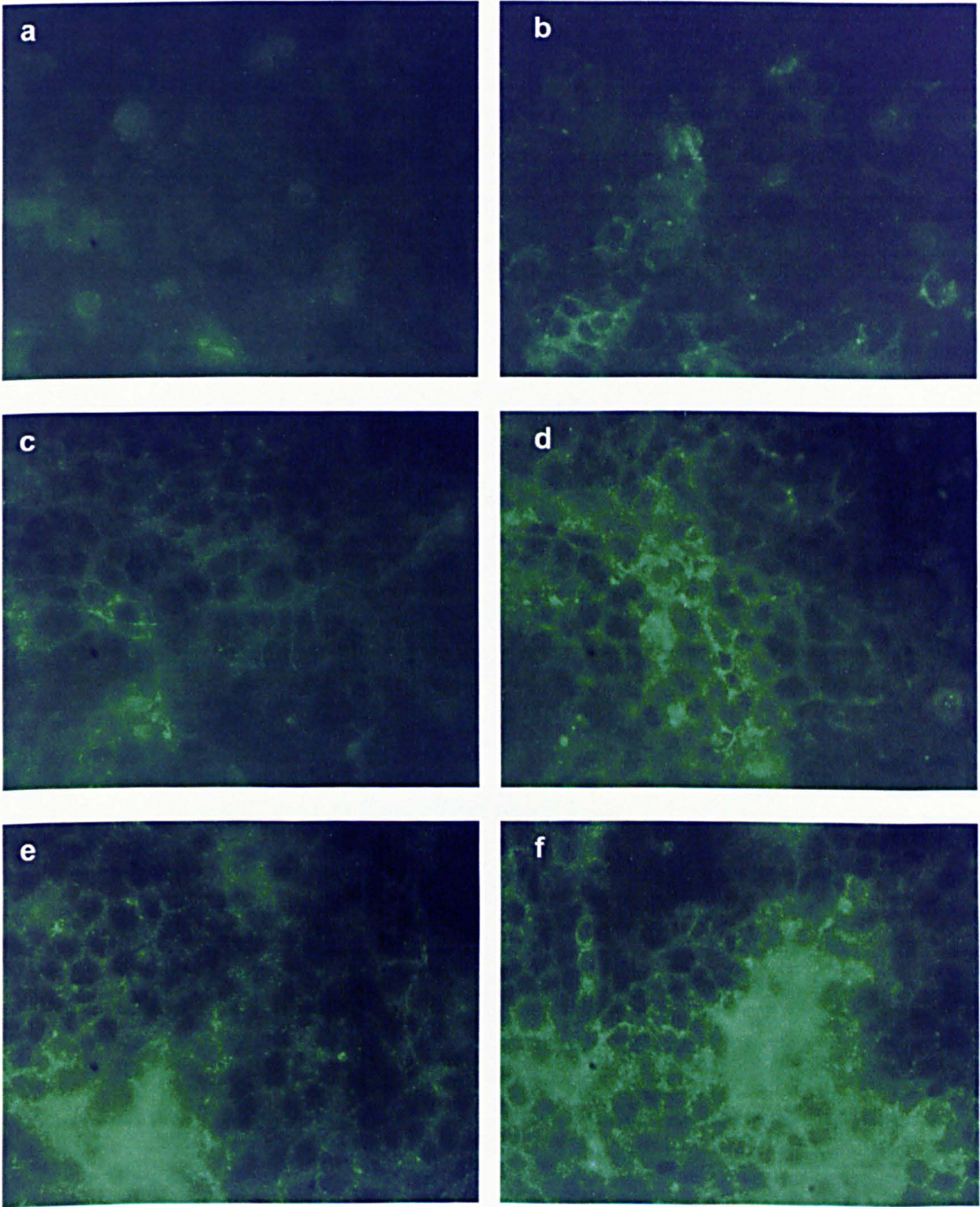
#### 4.9.4 Conclusions

A calcium concentration of at least  $500\mu\text{M}$  was required for the expression and correct intracellular localisation of occludin in J-HCET cells cultured in either HKGS or HKGS-V2 supplemented Epilife™ medium. Occludin expression and localisation appears to be similar to the control cultures at much lower calcium concentrations in Epilife™ medium supplemented with HKGS supplements. However, the possibility



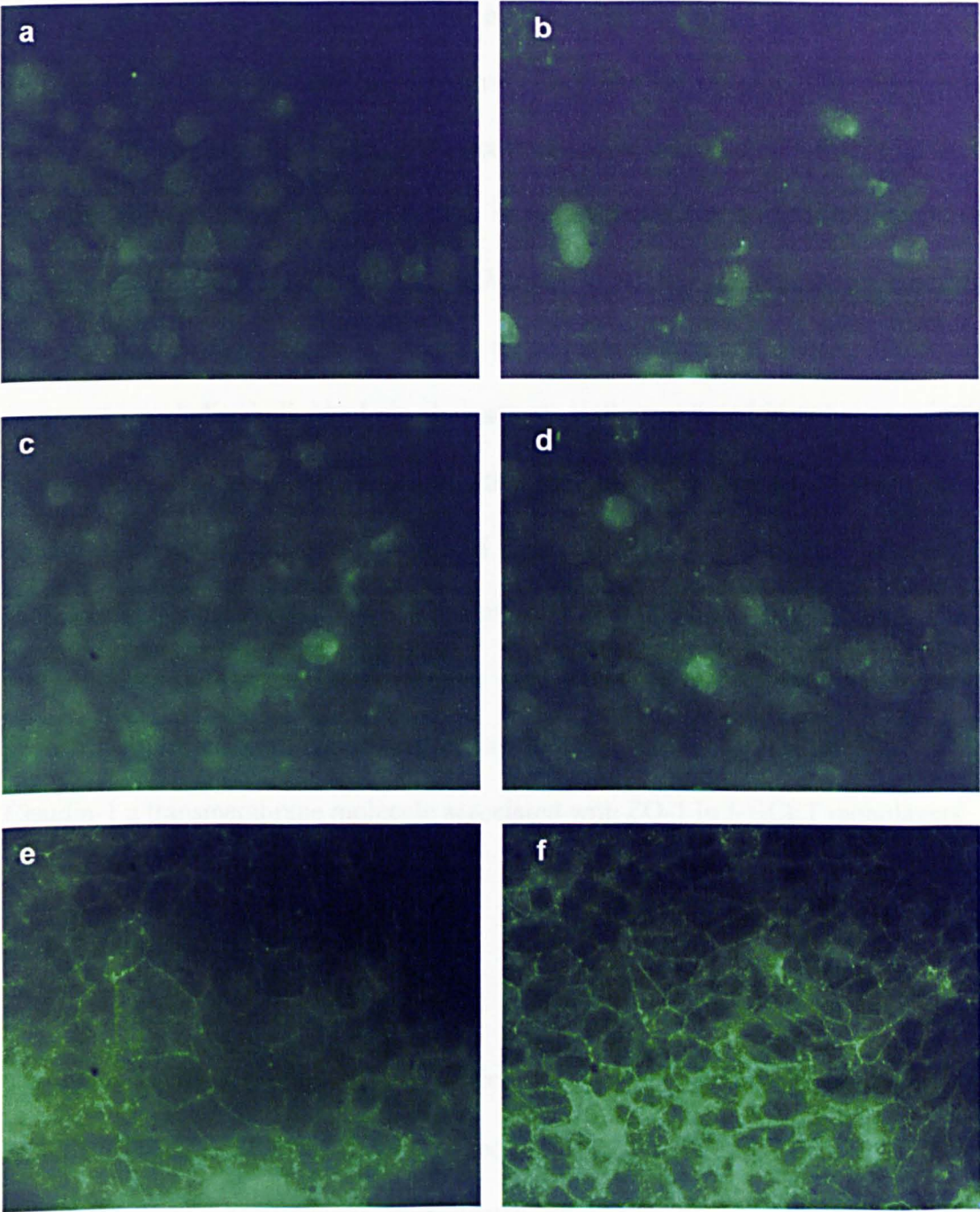
remains that calcium concentration could affect expression levels of this molecule. The focal pattern imposed upon the more diffuse membrane localisation at the higher calcium concentration would be consistent with occludin associated with the TJ via ZO-1 internally, but may be in excess of that which can be associated.

**Figure 4.48a/b/c/d/e/f:** Immunofluorescent staining of occludin in J-HCET monolayer cultured in HKGS supplemented Epilife media containing a) 0 b) 60 c) 80 d) 100 e) 500 and f) 1000 $\mu$ M calcium. (x400 magnification).





**Figure 4.49a/b/c/d/e/f:** Immunofluorescent staining of occludin in J-HCET monolayer cultured in HKGS-V2 supplemented Epilife media containing a) 0 b) 60 c) 80 d) 100 e) 500 and f) 1000 $\mu$ M calcium. (x400 magnification).



## **4.10 Modulation of Claudin-1 by growth media calcium concentration**

### **4.10.1 Introduction**

The barrier function is not only regulated by the membrane based molecules, but also those that have a paracellular cleft domain e.g. occludin and claudin. Claudin-1 and -2 were originally identified because they cofractionated with occludin from sonicated fractions (Furuse *et al.*, 1998). There is no evidence for a distinct interaction between occludin and claudin (Balda and Matter, 2000), and claudins do not copurify with occludin from detergent solubilised membranes (Balda and Matter, 2000). In contrast to occludin, individual claudins are generally expressed in only a restricted number of specific cell types, suggesting that they are associated with tissue-specific functions of tight junctions (Balda and Matter, 2000) but the level of claudin expression does not correlate with the number of tight junction strands (Saitou *et al.*, 1997; Tsukita and Furuse, 1999). Studies were conducted to determine the effects of growth medium calcium concentration has upon the expression and localisation of Claudin-1 a transmembrane molecule associated with ZO-1 in J-HCET monolayers.

### **4.10.2 Materials and Methods**

The same approach was taken as in section 4.8.2 except; the primary antibody was rabbit anti-claudin-1 (table 2.0) was diluted in 0.1% BSA (w/v) in PBS and incubated with the cells at 4°C overnight. Immunostaining of J-HCET monolayers cultured in the keratinocyte media (KGM) supplemented with animal derived products and calcium to give a final concentration of 100µM routinely used in the

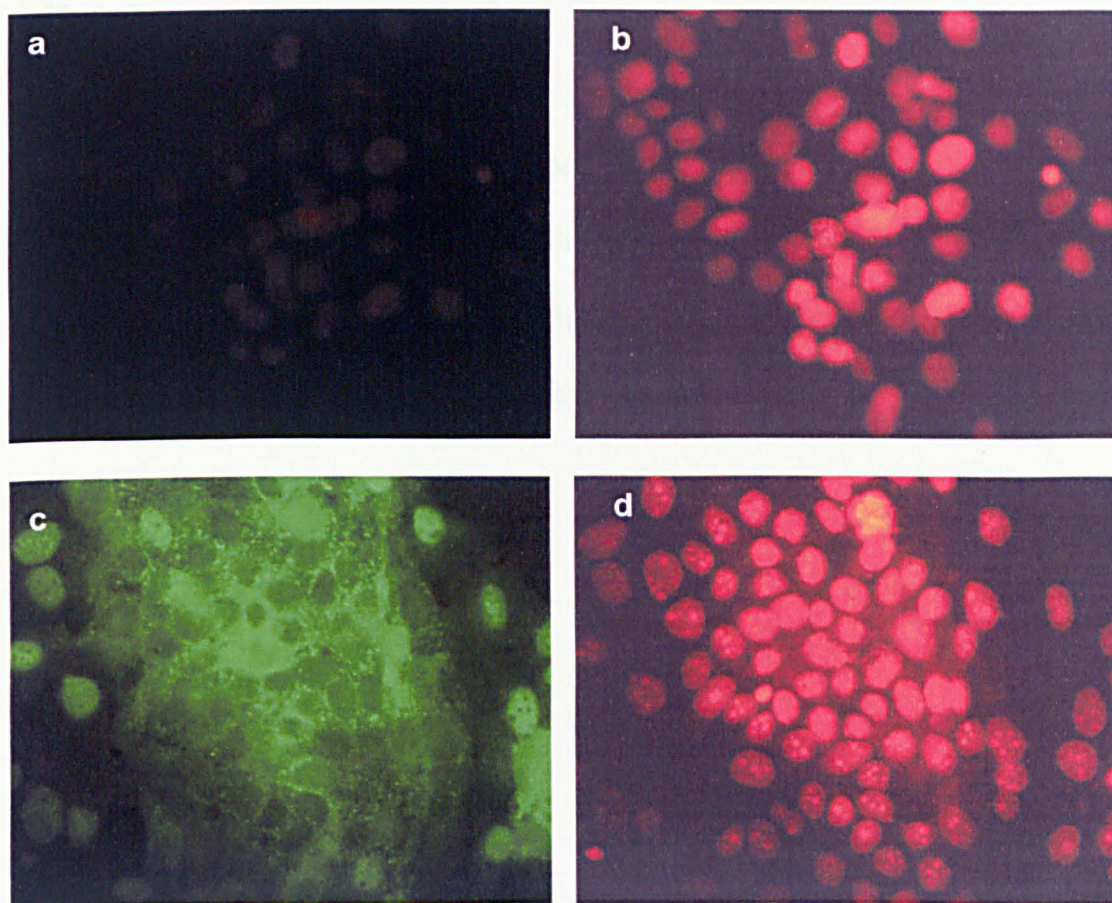
ICCVAM/ECVAM validation trial (Strickland *et al.*, 2003) was employed as a control (figure 4.50). Slides were viewed under a Leica Confocal Microscope.

#### **4.10.3 Results**

In control J-HCET monolayers the absence of Claudin-1 immunostaining (figure 4.50a) demonstrates no auto-fluorescence at the wavelengths used to visualise the FITC labelled anti-claudin-1 binding. The staining with propidium iodide confirmed the presence of a complete monolayer (figure 4.50b). The combined staining confirmed no cross fluorescence signal between claudin-1 and propidium iodide (figure 4.50c/d). Interaction with the control corneal cells in KGM with 100 $\mu$ M calcium revealed a beaded appearance along the cell/cell membrane contacts, but absent where one cell did not abut another (figure 4.50c).



**Figure 4.50a/b/c/d:** (a) KGM cultured J-HCET monolayer exposed to PBS instead of primary antibody. (b) The same J-HCET monolayer with propidium iodide staining. (c) KGM cultured J-HCET monolayer received rabbit anti-Claudin-1 and secondary antibody. (d) The same J-HCET monolayer with propidium iodide staining. (x400 magnification)



When the HKGS supplements were used, a calcium concentration of  $500\mu\text{M}$  was required (figure 4.51e) to generate a comparable distribution as observed for the KGM medium. Interestingly there also appears to be nuclear or cytoplasmic binding of this antibody at calcium concentrations of  $100\mu\text{M}$  and lower (figure 4.51a/b/c/d). A marked difference was observed with the corneal cell grown in HKGS-V2 supplemented medium. At calcium concentrations of  $100\mu\text{M}$  and below only nuclear

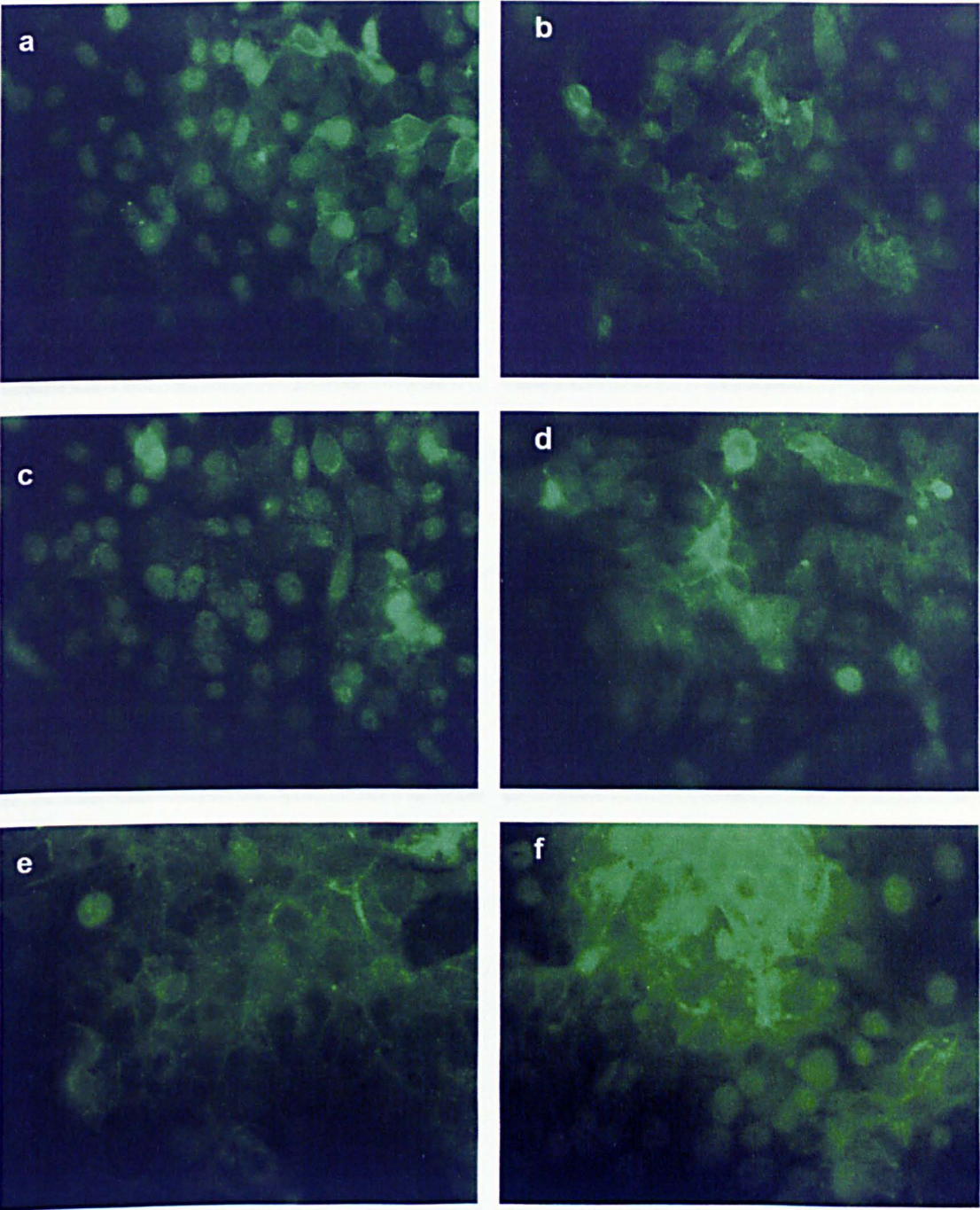


staining was observed (figure 4.52a/b/c/d). A calcium concentration of 500 $\mu$ M (figure 4.52e) was required to generate a similar distribution as observed for the KGM medium. In those cells expressing membrane staining, nuclear staining was absent, and vice versa. In contrast to both ZO-1 and occludin, claudin-1 was only correctly localised in areas where J-HCET monolayer had begun to multilayer.

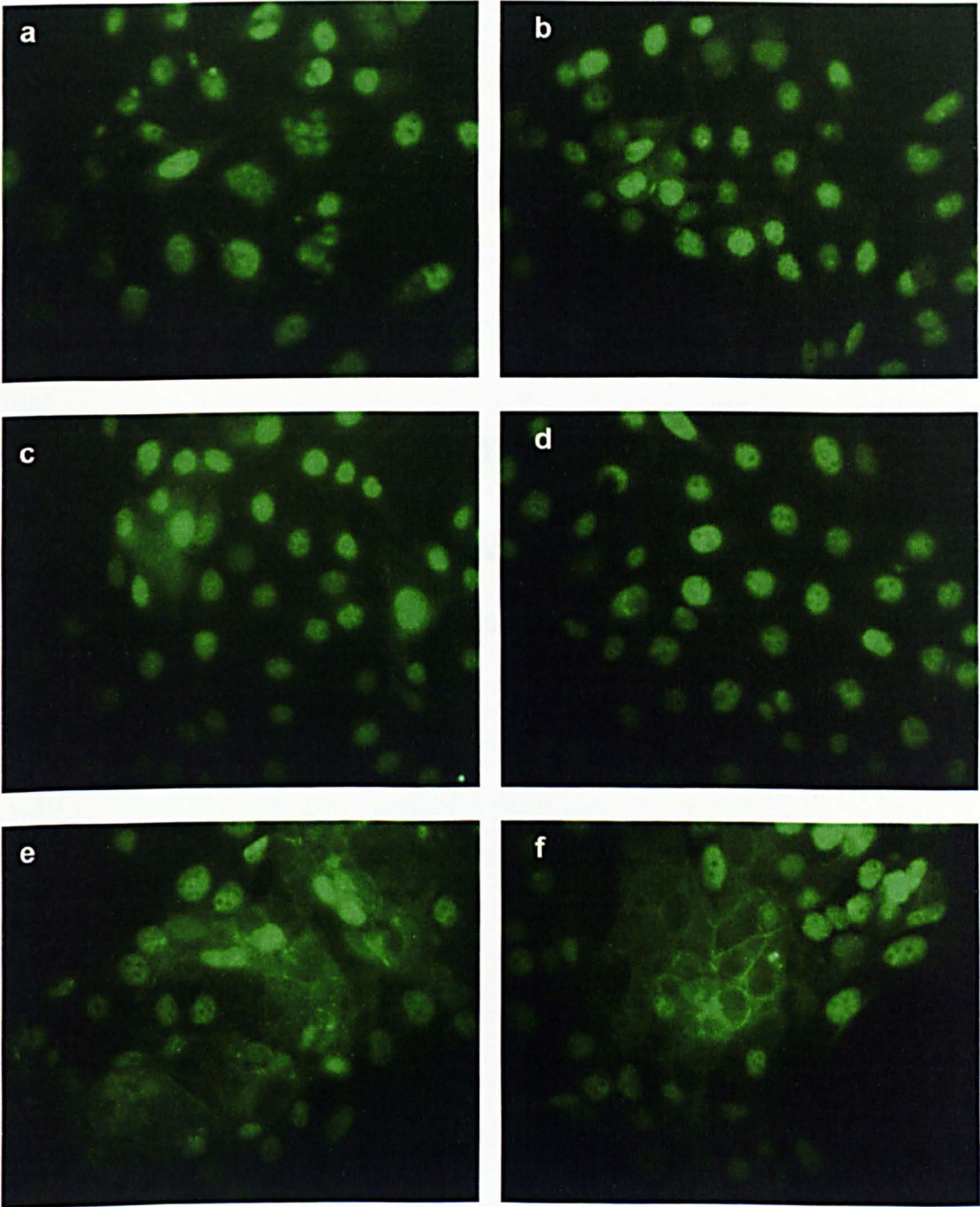
#### **4.10.4 Conclusions**

A calcium concentration of at least 500 $\mu$ M is required for the expression and correct localisation of claudin-1 in J-HCET cells cultured in either HKGS or HKGS-V2 supplemented Epilife™ medium. In addition, claudin-1 immunostaining in “hot spots” occurs only where J-HCET have begun to multilayer.

**Figure 4.51a/b/c/d/e/f:** Immunofluorescent staining of Claudin-1 in J-HCET monolayer cultured in HKGS supplemented Epilife™ media containing a) 0 b) 60 c) 80 d) 100 e) 500 and f) 1000 $\mu$ M calcium. (x400 magnification).



**Figure 4.52a/b/c/d/e/f:** Immunofluorescent staining of Claudin-1 in J-HCET monolayer cultured in HKGS supplemented Epilife™ media containing a) 0 b) 60 c) 80 d) 100 e) 500 and f) 1000 $\mu$ M calcium. (x400 magnification).



## **Chapter 4c**

### ***Effects of medium composition on barrier function***

**“Relating adhesion molecule expression and localisation to barrier function”**



# **Effects of medium composition on barrier function**

**“Relating adhesion molecule expression and localisation to barrier function”**

## **4.11 Effect of subcytotoxic surfactant exposure on f-actin, ZO-1, occludin and Claudin-1 in J-HCET cultured in HKGS supplemented media**

### **4.11.1 Introduction**

Although it has been demonstrated (section 4.4) that subcytotoxic surfactant exposure does not disrupt J-HCET barrier function or viability, the combined fluorescein leakage- resazurin reduction assay does not provide information as to the expression and localisation of the molecular components of the tight junction. In order to establish that chronic surfactant exposure does not alter the J-HCET tight junctions, the expression and localisation of f-actin, ZO-1, occludin and Claudin-1 were examined in monolayers cultured in medium containing subcytotoxic surfactant concentrations.

### **4.11.2 Materials and Methods**

Polycarbonate inserts were prepared as section 4.4 except the kenacid blue assay was not performed on all replicates. J-HCET cultures used for the initial total protein assay were aspirated and immunolabelled for f-actin, ZO-1 and Claudin-1 employing the same protocols as in sections 4.7, 4.8 and 4.10 respectively. For occludin immunostaining a similar approach was taken as in section 4.9 except that the

primary antibody was mouse anti-occludin (table 2.0). Immunostaining of J-HCET monolayers cultured in the keratinocyte medium (KGM) supplemented with animal derived products and calcium (100 $\mu$ M) routinely used in the ICCVAM/ECVAM validation trial (Strickland *et al.*, 2003) were employed as a control. Slides were viewed under a Leica Confocal Microscope. To visualise the cell-cell and cell matrix junctions, control J-HCET cultures were prepared for Transmission electron microscopy (TEM) (section 2.2.5.2). Samples were then viewed in a Phillips 410 Transmission Electron Microscope operated at 80KV.

### **4.11.3 Results**

#### **4.11.3.1 J-HCET viability and function**

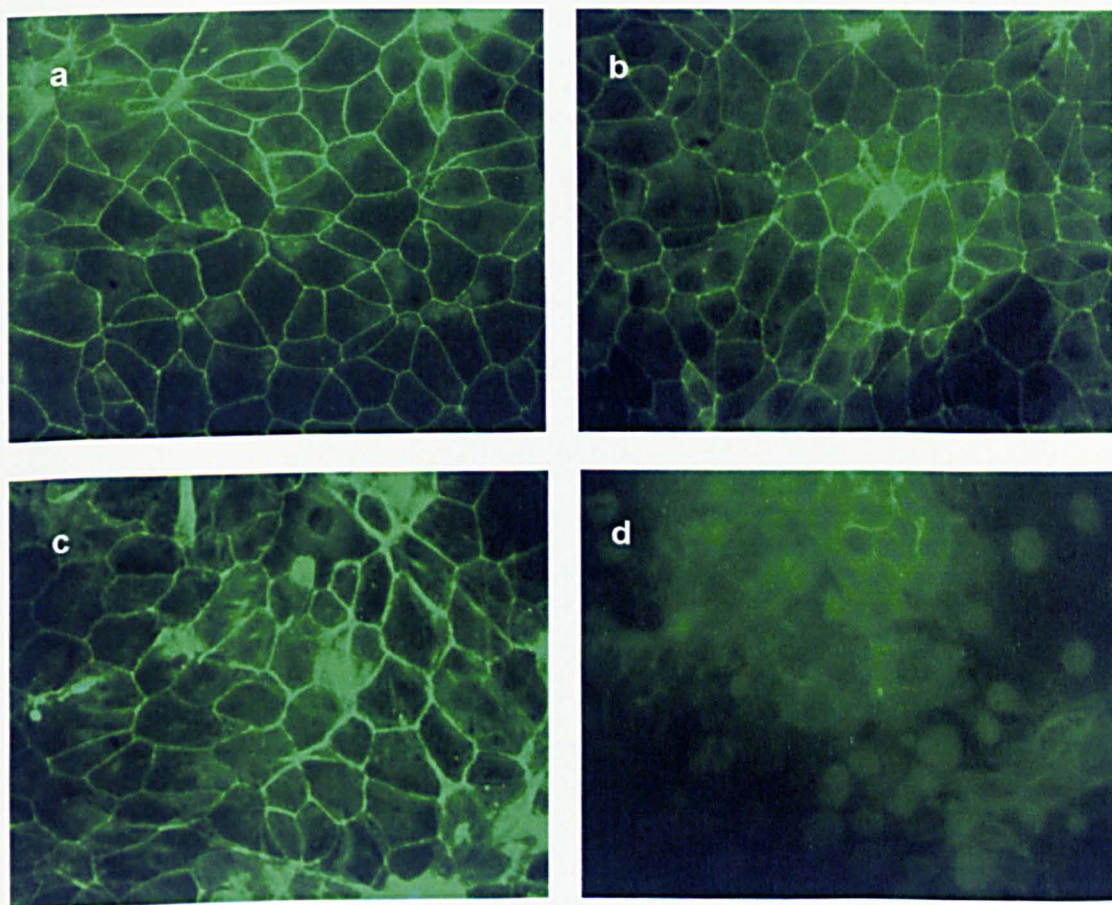
Subcytotoxic surfactant exposure of SDS; 4 $\mu$ gml<sup>-1</sup>, T20; 25 $\mu$ gml<sup>-1</sup>, CAPB 2.99 $\mu$ gml<sup>-1</sup> or BAK; 0.0025 $\mu$ gml<sup>-1</sup>) have no significant effect on barrier function (figure 4.21), activity (figure 4.22) or total cell protein (figure 4.23) or over 21 days (504 hours). At 504 hours no indications of pending adverse reactions were noted. The combined fluorescein leakage- resazurin reduction and kenacid blue assay are unable to demonstrate the expression and localisation of f-actin, ZO-1 and occludin.



#### 4.11.3.2 Expression and localisation of f-actin, ZO-1 and occludin

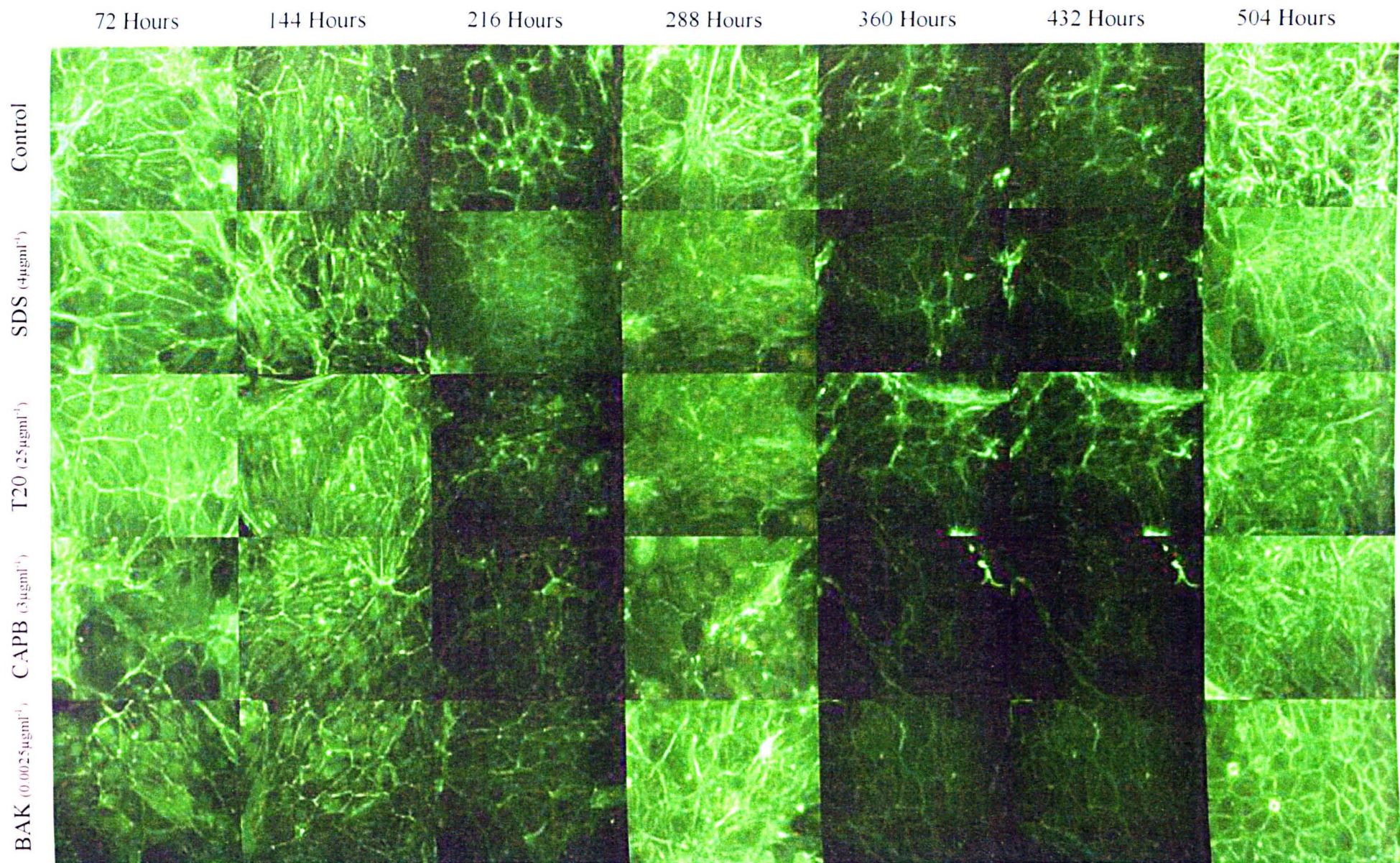
Immunohistochemistry controls were prepared as outlined in sections 4.7 through 4.9 for f-actin, ZO-1, and occludin and examined microscopically confirming the efficacy of the staining protocols. Cultures were examined prior to surfactant exposure to ensure the correct expression and localisation of f-actin, ZO-1, occludin and Claudin-1 (figure 4.56).

**Figure 4.56** : Images representative of (a) occludin, (c) ZO-1, (c) f-actin and (d) Claudin-1 expression in J-HCET monolayers cultured in HKGS supplemented medium prior to subcytotoxic surfactant exposure. (magnification x400).



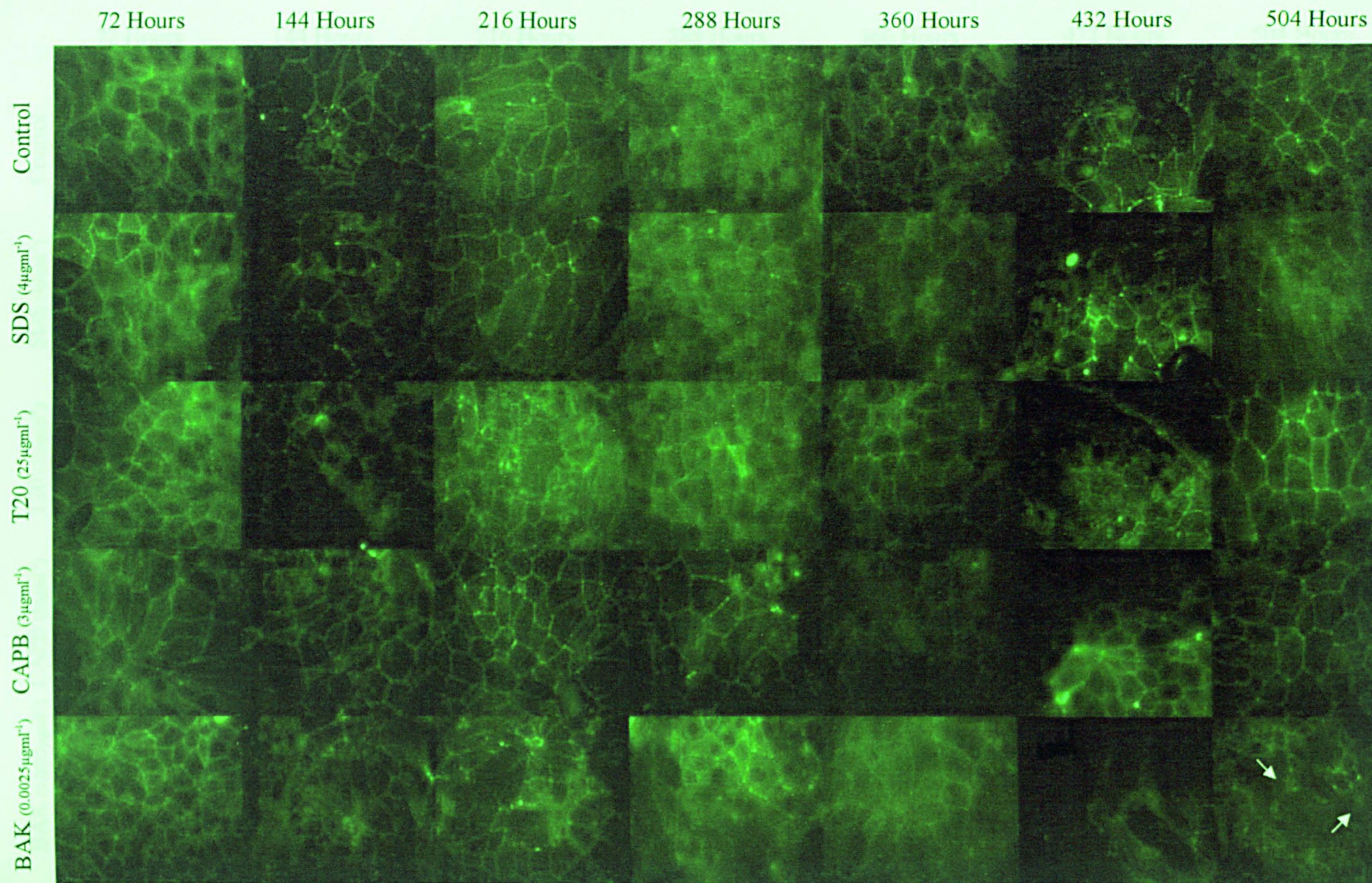
No observable alteration in the expression and localisation of f-actin (figure 4.57), ZO-1 (figure 4.58) or occludin (figure 4.59) occurred compared to control cultures after 432 hours of continuous exposure to subcytotoxic SDS, T20, CAPB or BAK concentrations. However, at 504 hours of continuous exposure to BAK ( $0.0025\mu\text{gml}^{-1}$ ) the expression pattern of ZO-1 compared to control cultures was significantly altered. The distinctive “chicken wire” pattern begins to degrade with a significant loss of fluorescence at abutting J-HCET membranes (figure 4.58). This cannot be attributed to cell loss as the total cell protein and viability of the cultures remain unaltered (figures 4.54 and 4.55). This alteration in ZO-1 expression and localisation is not associated with a significant increase in fluorescein leakage (figure 4.53). No significant alterations in the expression and localisation of f-actin or occludin are observed at similar time points (figures 4.57 and 4.59)





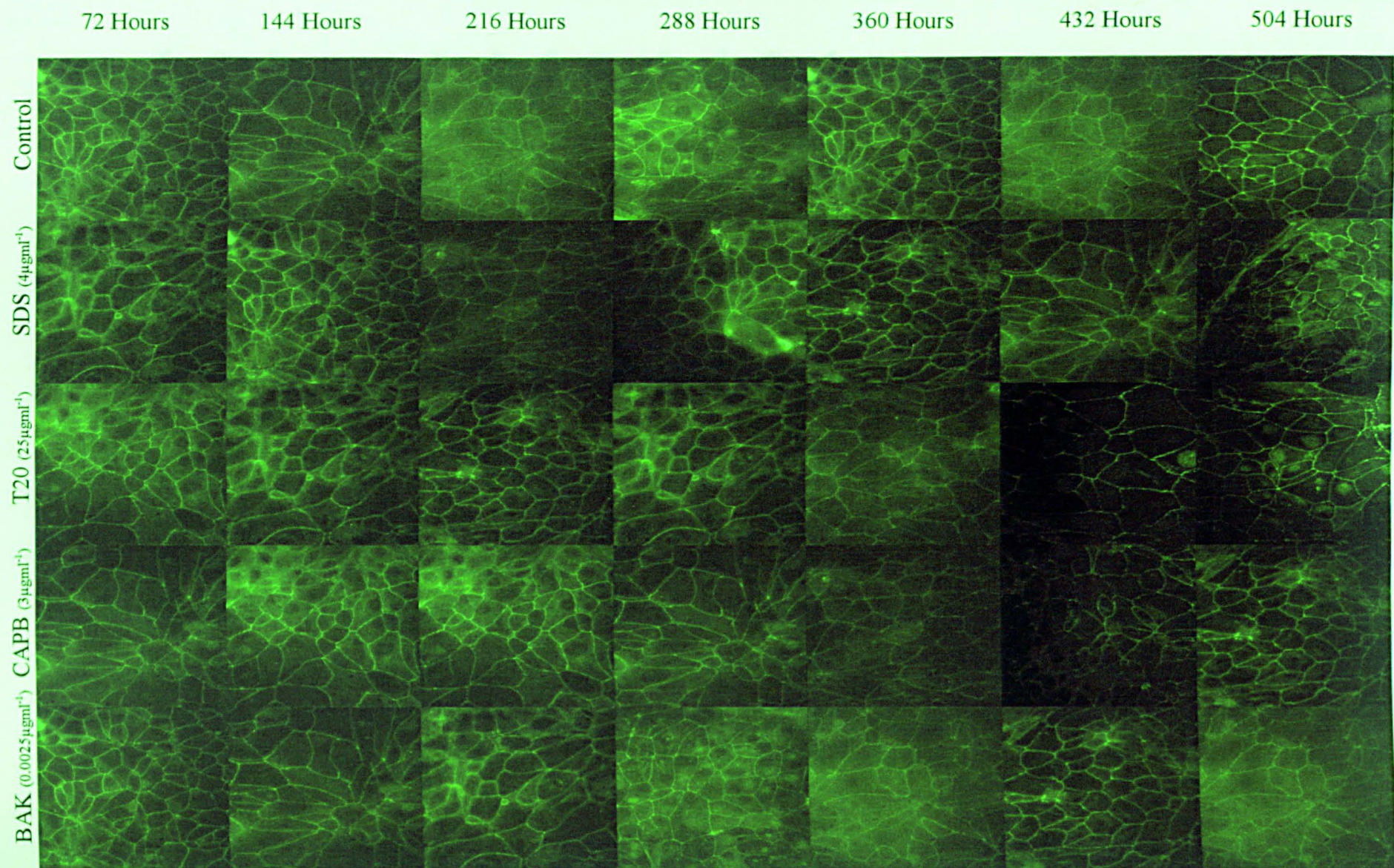
**Figure 4.57** : f-actin expression in J-HCET monolayers cultured in HKGS supplemented medium containing subcytotoxic concentrations of sodium dodecyl sulphate, tween 20, cocamidopropylbetaine or benzalkonium chloride over 504 hours in continuous culture. (magnification x400).





**Figure 4.58** : ZO-1 expression in J-HCET monolayers cultured in HKGS supplemented medium containing subcytotoxic concentrations of sodium dodecyl sulphate, tween 20, cocamidopropylbetaine or benzalkonium chloride over 504 hours in continuous culture. (magnification x400).



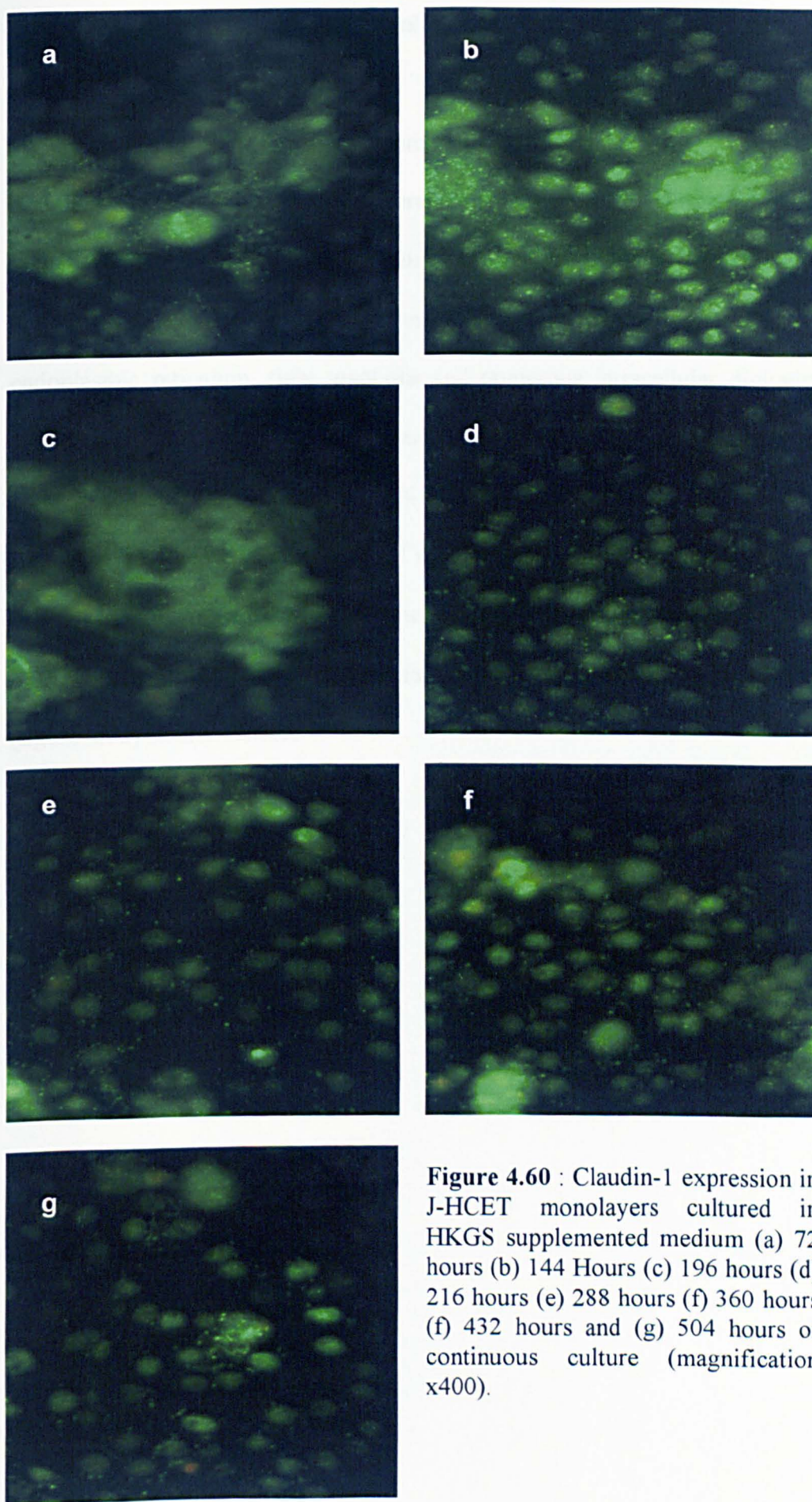


**Figure 4.59** : Occludin expression in J-HCET monolayers cultured in HKGS supplemented medium containing subcytotoxic concentrations of sodium dodecyl sulphate, tween 20, cocamidopropylbetaine or benzalkonium chloride over 504 hours in continuous culture. (magnification  $\times 400$ ).

#### 4.11.3.3 Expression and localisation of Claudin-1

Although it was demonstrated that Claudin-1 expression and localisation is dependent upon a medium calcium concentration of 1mM (section 4.10) it was also observed that immunostaining only occurs in “hot spots” where J-HCET have begun to multilayer. This is in contrast to other components of the tight junction f-actin, ZO-1 and occludin which are expressed and correctly localised in monolayers. Claudin-1 immunostaining of the control J-HCET cultures reveal a generalised pattern of nuclear staining (figure 4.60) i.e. as seen in figure 4.51d. Although claudin-1 has been reported to be important in the barrier function of the human cornea (Ban *et al.*, 2003) the leakage of fluorescein (figure 4.53) across the J-HCET cultures is within the defined limits (Clothier and Samson, 1996) indicative of an intact barrier. Of the total area available for cellular attachment on the polycarbonate insert, less than 5% demonstrate J-HCET multilayering and so therefore the role claudin-1 plays in barrier function of this human cornea equivalent *in vitro* is unclear.



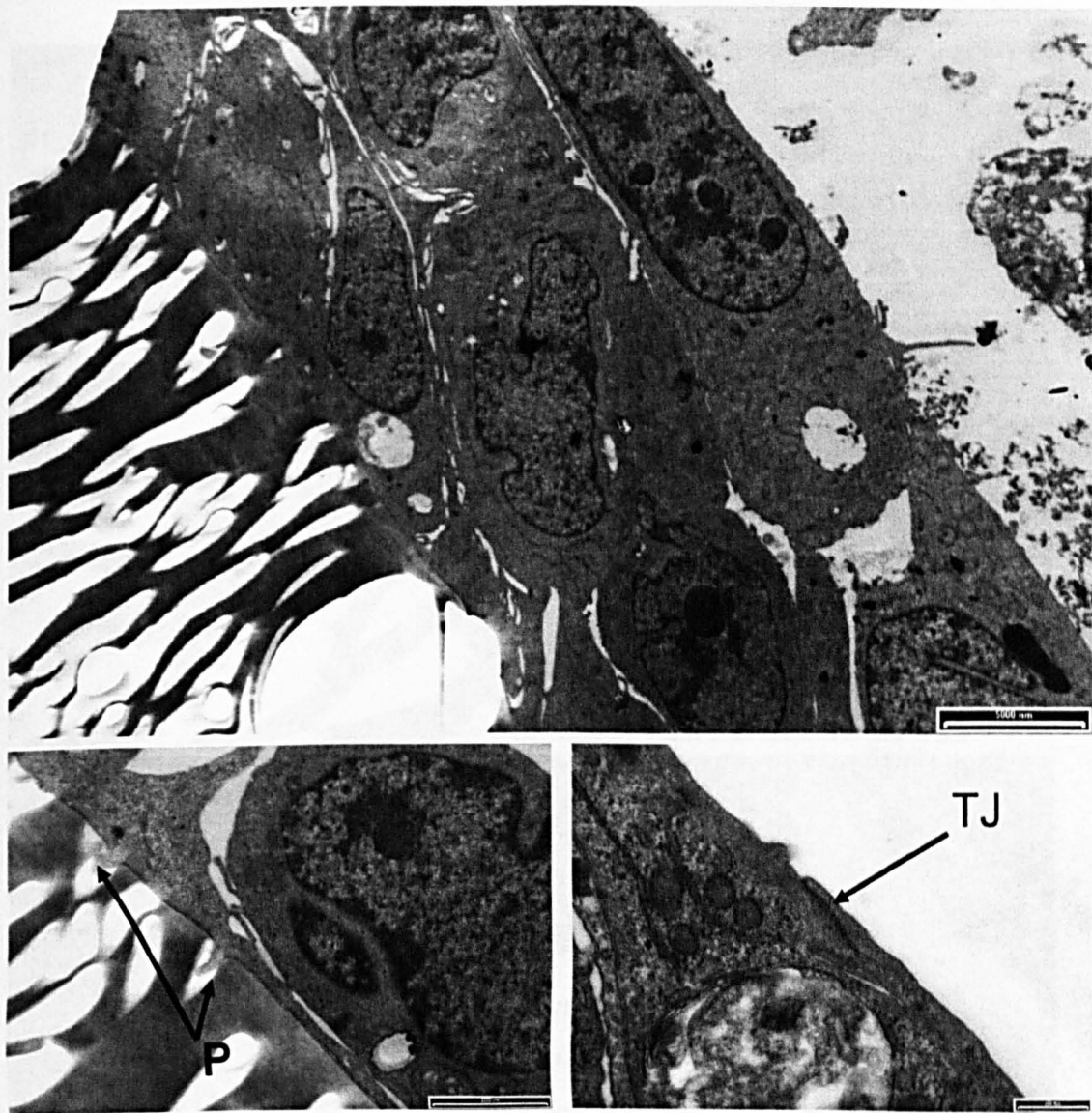


**Figure 4.60 :** Claudin-1 expression in J-HCET monolayers cultured in HKGS supplemented medium (a) 72 hours (b) 144 Hours (c) 196 hours (d) 216 hours (e) 288 hours (f) 360 hours (f) 432 hours and (g) 504 hours of continuous culture (magnification x400).

#### **4.11.3.4 Transmission electron microscopy of J-HCET junctions**

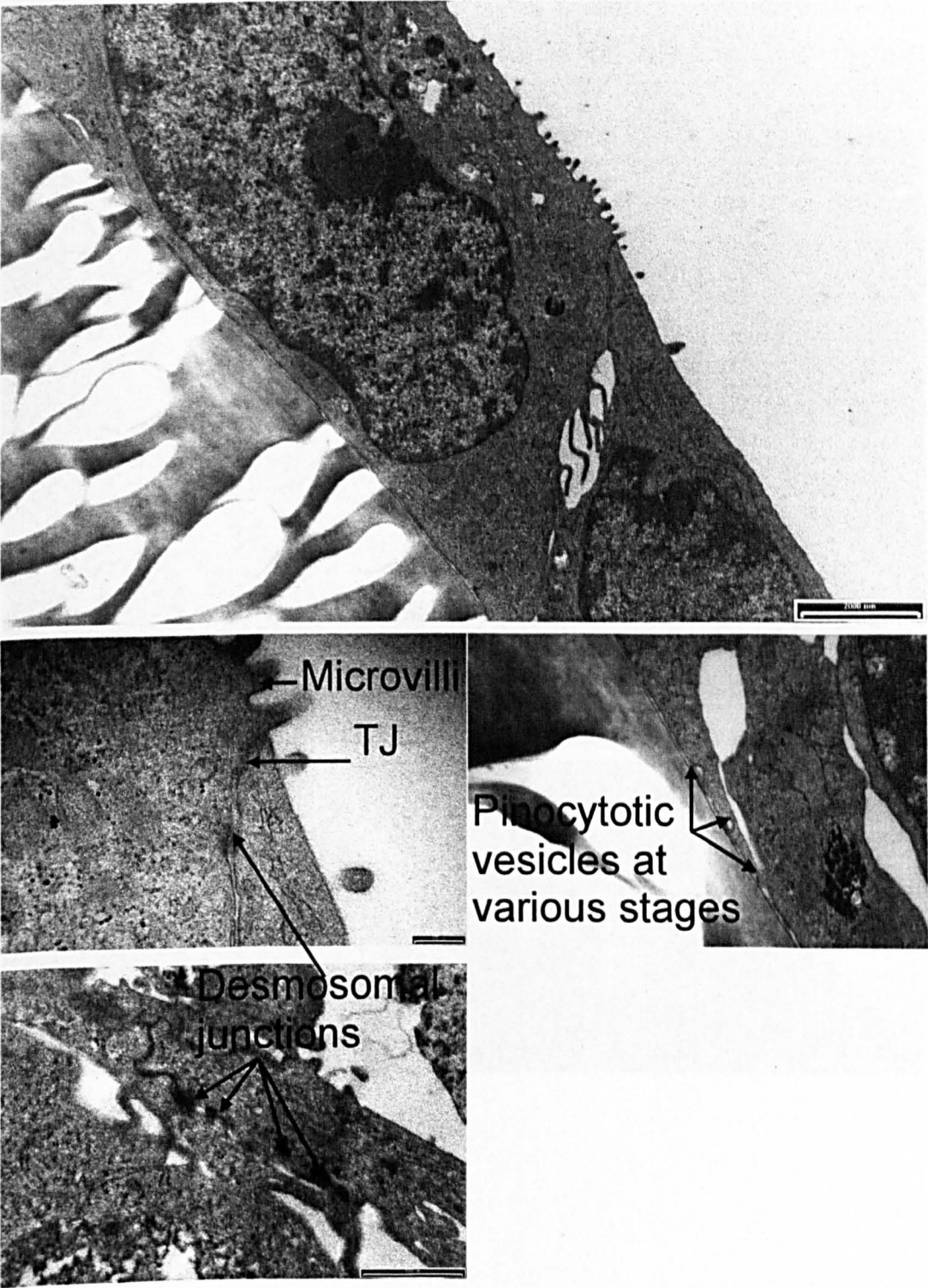
To gain insight into the ultrastructure of the tight junctions, in addition to immunostaining, J-HCET cultures were evaluated by TEM. Ultrastructure features common to the human corneal epithelium are observed in the J-HCET model. These include surface cell microvilli, nuclear membranes, mitochondria, Golgi bodies, endoplasmic reticulum, tight junctions and numerous intracellular digitations and desmosomes. Tight junctions are clearly visible at the apical domain of abutting J-HCET in both multilayer (figure 4.61) and monolayer (figure 4.62) regions respectively. The close interaction of the abutting J-HCET membranes in the tight junctions were confirmed by tilting the TEM grid obtaining images at  $\pm 15^\circ$  (figure 4.63). Numerous desmosome junctions were observed with pinocytotic vessels (figure 4.62) clearly visible.

**Figure 4.61 :** Multilayer region of the J-HCET cells showing projections into the polycarbonate insert pores (P), and tight junctions at the apex (TJ).

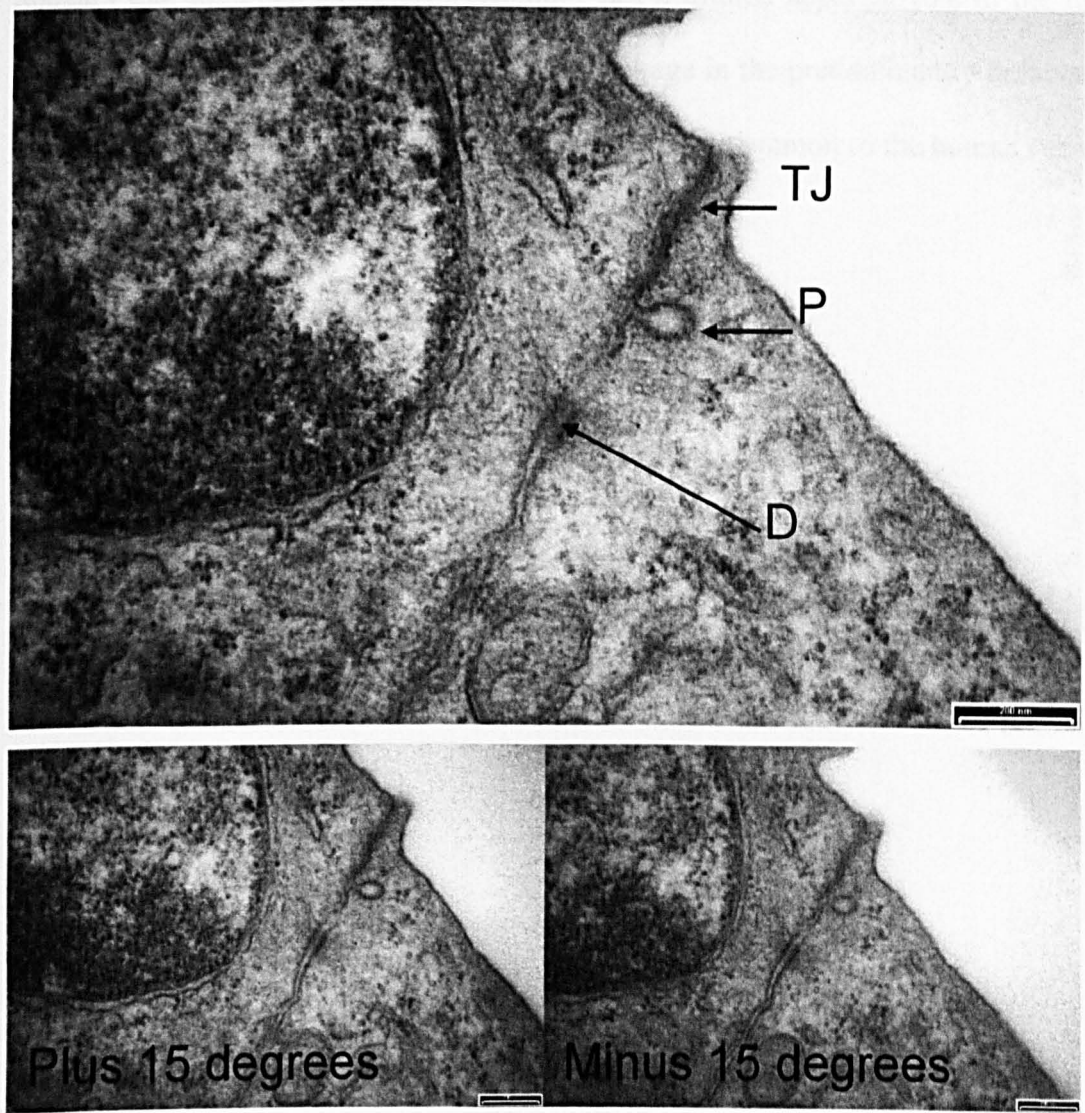




**Figure 4.62 :** Monolayer region of the J-HCET cells showing TJ's, desmosomal junctions and pinocytotic vesicles at both apical and basal regions.



**Figure 4.63 :** To confirm the presence of the tight junctions the image was tilted plus or minus 15 degrees. The close approximation of the junction was still observed. Note the pinocytotic vesicle (P) adjacent to the tight junction (TJ) and desmosomal junction (D).



#### **4.11.4 Conclusions**

Subcytotoxic benzalkonium chloride exposure appears to disrupt ZO-1 expression and localisation after 432 hours of continuous exposure. No alterations are observed in the expression and localisation of f-actin or occludin. Although it has been reported (Ban *et al.*, 2003) that claudins are involved tight junction formation it appears that although expressed, claudin-1 has a limited apparent role in the tight junctional prevention of sodium fluorescein leakage in the predominantly monolayer model employed in this study. Ultrastructure features common to the human corneal epithelium are observed in the J-HCET model.



## **Chapter 5**

### ***Effects of chronic Timolol maleate exposure***

# Effects of chronic Timolol maleate exposure

## 5.1 Maximal concentration of buthionine sulphoxamine required to reduce glutathione levels with no alteration of J-HCET viability

### 5.1.1 Introduction

Since Timolol maleate is detoxified *in vivo* via conjugation with glutathione (GSH) (Megaw, 1984; Riley, 1990) is important to establish that a similar mechanism occurs *in vitro*. Buthionine sulphoxamine (BSO) is known to reduce GSH (Mulder and Ouwerkerek-Mahadevan, 1997; Pastore *et al.*, 2003). Since timolol maleate containing eye drops can be preserved with benzalkonium chloride and used repeatedly for glaucoma, it is possible that its use could affect the corneal epithelial levels of GSH. BSO was used to confirm if timolol maleate toxicity could be affected *in vitro* in human corneal cells following reduction in available GSH.

### 5.1.2 Materials and Methods

Immediately prior to use, buthionine sulphoxamine was completely dissolved in growth media (section 2.1.7.1) to create concentrations of 0.5, 1, 5, 10, 50, 100, and 500 $\mu$ M. J-HCET seeded into the central 54 wells of two 96 well plates, 100 $\mu$ l of  $3 \times 10^5$  cells ml<sup>-1</sup>. Phosphate buffered saline (100 $\mu$ l) was added to the outer 36 wells to prevent excessive evaporation of growth medium. Plates were incubated for 72 hours at 37°C and 5% CO<sub>2</sub> (v/v) in air to allow the cells to attach and attain confluence. One plate was then assayed to provide a measure of the pre-exposure

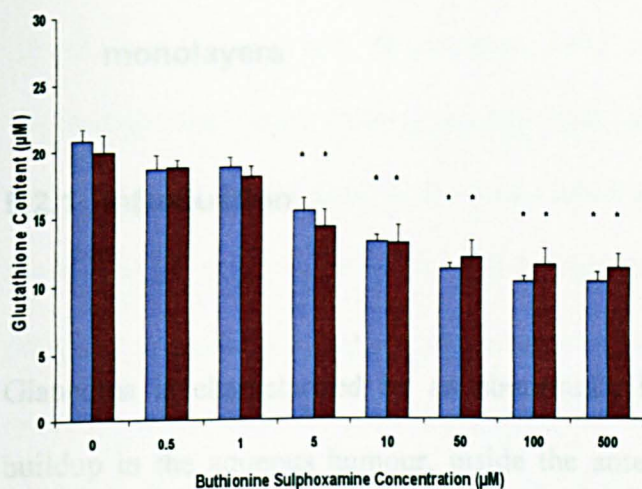
control levels. Medium was aspirated and the NRU (section 2.2.4.2), glutathione equivalents (section 2.2.4.3) and KB (section 2.2.4.1) assays performed. The medium was aspirated from the remaining plate and 200µl of one of each of the BSO concentrations applied as appropriate. Plates were incubated at 37°C and 5% CO<sub>2</sub> (v/v) in air for 48 hours. The neutral red uptake assay and the glutathione equivalents/kenacid blue assays were performed on the same cultures.

### **5.1.3 Results**

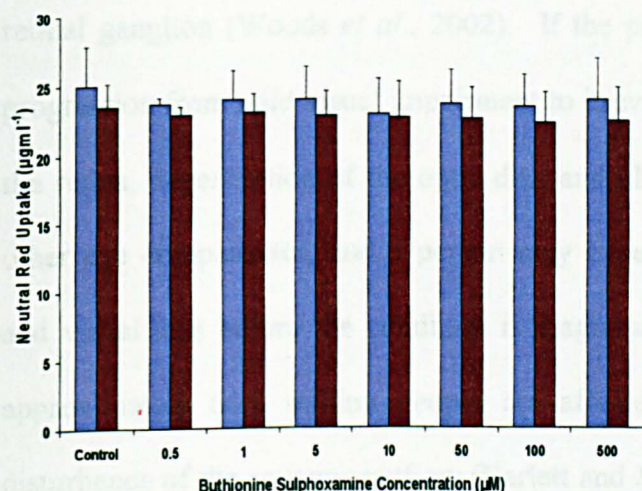
Over a 48 hour period the presence of at least 50µM BSO was required to reduce glutathione by 50% (figure 5.01) whilst not affecting cell viability (figure 5.02) or total cell number (figure 5.03). Between 50 and 500µM the level of reduction in GSH is not enhanced.

### **5.1.4 Conclusion**

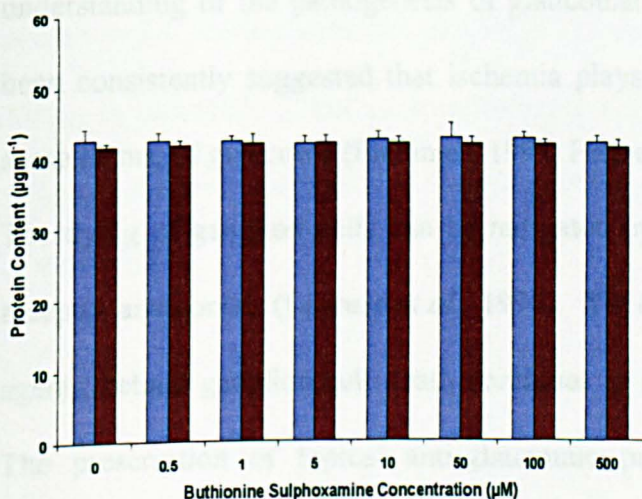
BSO can be used to reduce the GSH levels by 50% at a non-cytotoxic concentration. This was a desired reduction since it allows for the measuring of further reduction upon timolol maleate exposure, and/or timing of recovery to normal level with the BSO removal.



**Figure 5.01** : Glutathione content ( $\mu\text{M}$ ) of J-HCET monolayers cultured in ■ HKGS or ■ HKGS-V2 supplemented medium containing buthionine sulfoxamine for 48 hours. Where \* denotes a significant ( $P < 0.05$ ) decrease in glutathione content. (mean+S.D.,  $n=3$ ).



**Figure 5.02** : Neutral red uptake ( $\mu\text{gml}^{-1}$ ) of J-HCET monolayers cultured in ■ HKGS or ■ HKGS-V2 supplemented medium containing buthionine sulfoxamine for 48 hours. (mean+S.D.,  $n=3$ ).



**Figure 5.03** : Protein content ( $\mu\text{gml}^{-1}$ ) of J-HCET monolayers cultured in ■ HKGS or ■ HKGS-V2 supplemented medium containing buthionine sulfoxamine for 48 hours. (mean+S.D.,  $n=3$ ).

## **5.2 The effects of acute Timolol Maleate exposure on J-HCET monolayers**

### **5.2.1 Introduction**

Glaucoma is characterised by an abnormally high intraocular pressure due to a buildup in the aqueous humour, inside the anterior chamber of the eye. The fluid compresses the lens into the vitreous body and puts pressure on the neurons of the retinal ganglion (Woods *et al.*, 2002). If the pressure continues to rise, there is a progression from mild visual impairment to irreversible destruction of the neurons of the retina, degeneration of the optic disk and blindness. Glaucoma is painless, the other eye compensates, and a person may experience considerable retinal damage and visual loss before the condition is diagnosed. It is estimated that worldwide, approximately 66.8 million people are affected by primary glaucoma, a direct disturbance of the aqueous outflow (Barlett and Jaanus, 2001). Although our current understanding of the pathogenesis of glaucomatous optic neuropathy is poor it has been consistently suggested that ischemia plays a role in the aetiology, of at least some forms, of glaucoma (Flammer, 1994; Hayreh *et al.*, 1999; Osborne *et al.*, 2004). The dying of ganglion cells can be mitigated by intraocular injection of glutamate receptor antagonists (Lagreze *et al.*, 1998). Yet despite treatment with IOP lowering agents, retinal ganglion cell death continues in many patients (Wood *et al.*, 2002). The prescription of topical anti-glaucomic preparations is standard for newly diagnosed patients (Barlett and Jaanus, 2001). The  $\beta$ -adrenoceptor antagonists, which comprise selective  $\beta_1$  (e.g. betaxol) and non-selective  $\beta_1/\beta_2$  (e.g. timolol) adrenoceptor antagonists, are one of the most important classes of pharmaceuticals

used to clinically lower elevated intraocular pressure (IOP) in glaucoma patients (Brooks and Gillies, 1992; Zimmerman, 1993; Wood *et al.*, 2002).  $\beta$  adrenoceptors are widely distributed throughout the body and are subdivided into  $\beta_1$  and  $\beta_2$  receptors according to their location and functions.  $\beta_1$  receptors are predominant in the heart and those found in the eye control the formation of aqueous humour  $\beta_2$  receptors predominate in other organs such as the lung, peripheral blood vessels and skeletal muscle. This is a clinically useful subclassification but is not absolute: for example there are  $\beta_2$  receptors in the heart and  $\beta_1$  receptors in the kidney (Zimmerman, 1993). Timolol maleate (TM) is one of the drugs of choice for treatment of glaucoma (Uusitalo *et al.*, 1998; Aggarwal and Kaur, 2005). Since excessive loss of timolol maleate through nasolacrimal drainage can cause respiratory and cardiovascular side effects (Everitt and Avorn, 1990). Timolol maleate has the potential to have adverse side effects on the corneal epithelium. Whilst standard surfactants are good indicators of the effects following chronic exposure, there are pharmaceuticals designed for chronic/repeat use in the eye. Preliminary studies were carried out to determine the amount of damage caused by acute timolol maleate exposure to J-HCET monolayers. This was assessed using the resazurin reduction, glutathione equivalents and kenacid blue assays. The normal eye drop solutions preserved with BAK contains timolol maleate at a concentration of  $2.5\text{mgml}^{-1}$ .



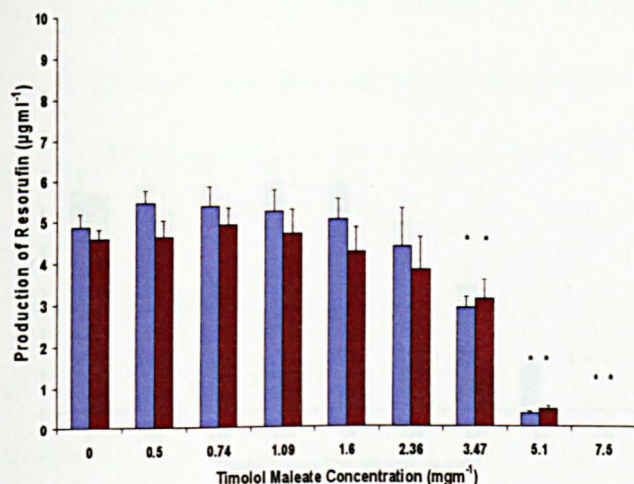
### 5.1.2 Materials and Methods

Immediately prior to use, timolol maleate was completely dissolved in HBSS (containing 1mM calcium chloride) to create concentrations of 0.5, 0.74, 1.09, 1.6, 2.36, 3.47, 5.1 and 7.5mgml<sup>-1</sup>. J-HCET were seeded at  $3 \times 10^5$  cells ml<sup>-1</sup> (100μl) per 54 central wells. Phosphate buffered saline (100μl) was applied to the outer 36 wells to prevent excessive evaporation of growth medium. Plates were incubated for 72 hours at 37°C and 5% CO<sub>2</sub> (v/v) in air to allow the cells to attach and attain confluence. One plate was assayed following the 72 hour growth period, providing a pre-exposure control. Medium was aspirated and the resazurin reduction (section 2.2.4.2), glutathione equivalents (section 2.2.4.3) and kenacid blue (section 2.2.4.1) assays performed. The medium was aspirated from the remaining plates and 200μl of each of the timolol maleate concentrations in HBSS or HBSS alone applied for 60 seconds. Wells were aspirated and the monolayers washed twice with 200μl of HBSS (60 seconds) containing 1mM calcium chloride. HBSS was aspirated and 200μl of cell culture medium (pre-warmed to 37°C) applied. Plates were incubated at 37°C and 5% CO<sub>2</sub> (v/v) in air for 48 hours. The resazurin reduction, glutathione equivalents and kenacid blue assays were performed on the remaining plate. The experiment was repeated with J-HCET co-exposed to 50μM BSO.

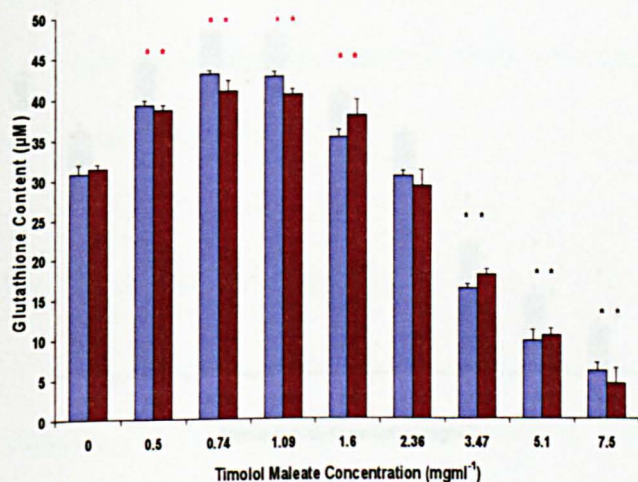
### 5.1.3 Results

Low concentrations of timolol maleate (0.5mgml<sup>-1</sup> to 1.6mgml<sup>-1</sup>) significantly ( $P < 0.05$ ) increased the levels of intracellular GSH (figure 5.05), with no significant alterations in either resorufin production (figure 5.04) or total cell protein (figure

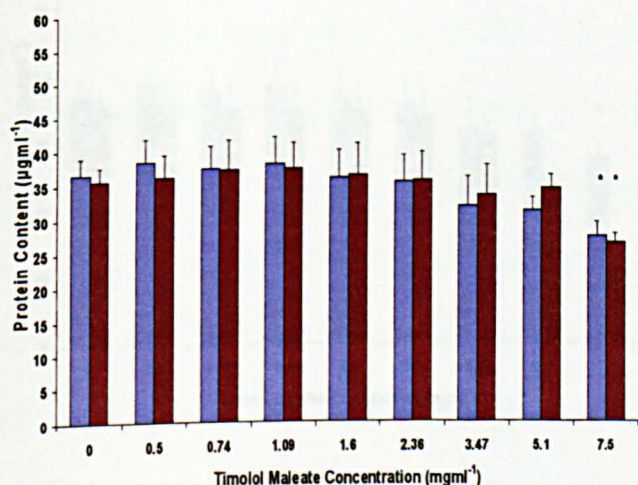
5.06). Timolol maleate, at a concentration of  $3.47\text{mgml}^{-1}$ , significantly reduced cell activity (figure 5.04) and GSH concentration (figure 5.05) with no alteration in total culture protein (figure 5.06). At a concentration of  $7.5\text{mgml}^{-1}$ , cell activity, GSH concentration and total cell protein were significantly reduced (figure 5.04, 5.05 and 5.06). When J-HCET monolayers were co-exposed to  $50\mu\text{M}$  BSO, the stimulatory effects on intracellular GSH, of the timolol maleate concentration was negated. Cell activity (figure 5.07) was significantly reduced at concentrations between  $1.09\text{mgml}^{-1}$  and  $7.5\text{mgml}^{-1}$  with corresponding significant decreases in GSH (figure 5.08) compared to control cultures. At timolol concentrations of 5.1 and  $7.5\text{mgml}^{-1}$  the total cell protein was significantly reduced (figure 5.09).



**Figure 5.04 :** Resorufin production ( $\mu\text{gml}^{-1}$ ) of J-HCET monolayers cultured in medium containing either  $\blacksquare$  HKGS or  $\blacksquare$  HKGS-V2 supplements exposed to Timolol Maleate concentrations for 48 hours. Where \* denotes a significant ( $P<0.05$ ) decrease in resorufin compared to control ( $0\text{mgml}^{-1}$ ) cultures. (mean+S.D.,  $n=3$ ).

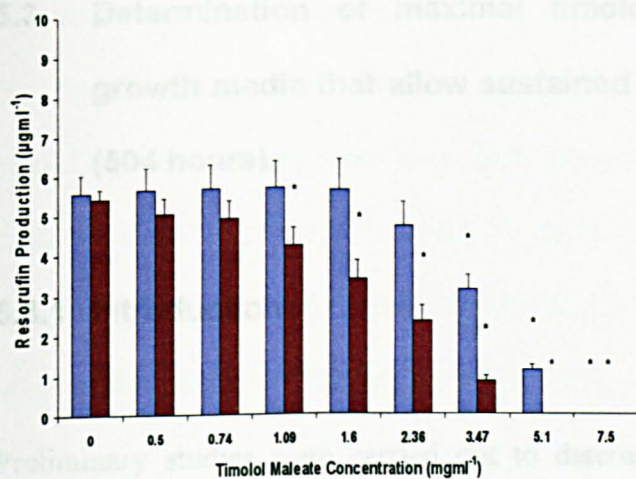


**Figure 5.05 :** Glutathione content ( $\mu\text{M}$ ) of J-HCET monolayers cultured in medium containing either  $\blacksquare$  HKGS or  $\blacksquare$  HKGS-V2 supplements exposed to Timolol Maleate concentrations for 48 hours. Where \* denotes a significant ( $P<0.05$ ) decrease and \* a significant increase in glutathione content compared to control ( $0\text{mgml}^{-1}$ ) cultures. (mean+S.D.,  $n=3$ ).

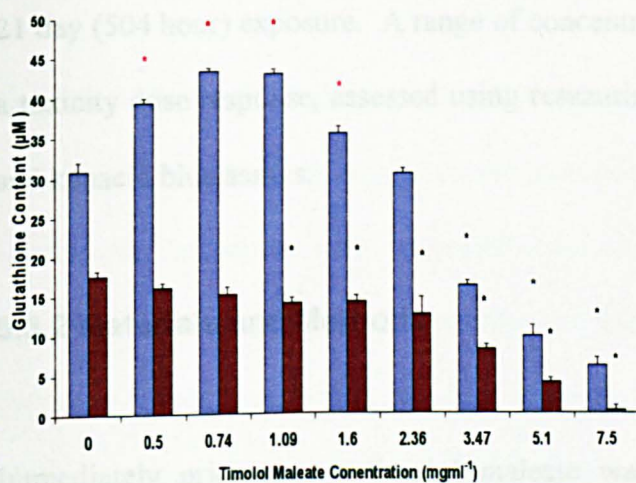


**Figure 5.06 :** Protein content ( $\mu\text{gml}^{-1}$ ) of J-HCET monolayers cultured in medium containing either  $\blacksquare$  HKGS or  $\blacksquare$  HKGS-V2 supplements exposed to Timolol Maleate concentrations for 48 hours. Where \* denotes a significant ( $P<0.05$ ) decrease in protein content compared to control ( $0\text{mgml}^{-1}$ ) cultures. (mean+S.D.,  $n=3$ ).

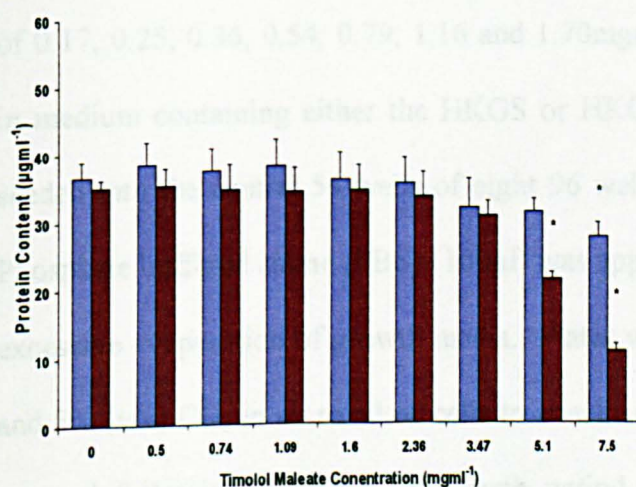




**Figure 5.07 :** Resorufin production ( $\mu\text{gml}^{-1}$ ) of J-HCET monolayers cultured in medium containing  $\blacksquare$  HKGS or  $\blacksquare$  HKGS and BSO (100 $\mu\text{M}$ ) exposed to Timolol Maleate concentrations for 48 hours. Where \* denotes a significant ( $P<0.05$ ) decrease in resorufin compared to control (0 $\text{mgml}^{-1}$ ) cultures. (mean+S.D.,  $n=3$ ).



**Figure 5.08 :** Glutathione content ( $\mu\text{M}$ ) of J-HCET monolayers cultured in medium containing  $\blacksquare$  HKGS or  $\blacksquare$  HKGS and BSO (100 $\mu\text{M}$ ) exposed to Timolol Maleate concentrations for 48 hours. Where \* denotes a significant ( $P<0.05$ ) decrease and \* a significant increase in glutathione content compared to control (0 $\text{mgml}^{-1}$ ) cultures. (mean+S.D.,  $n=3$ ).



**Figure 5.09 :** Protein content ( $\mu\text{gml}^{-1}$ ) of J-HCET monolayers cultured in medium containing  $\blacksquare$  HKGS or  $\blacksquare$  HKGS and BSO (100 $\mu\text{M}$ ) exposed to Timolol Maleate concentrations for 48 hours. Where \* denotes a significant ( $P<0.05$ ) decrease in protein content compared to control (0 $\text{mgml}^{-1}$ ) cultures. (mean+S.D.,  $n=3$ ).

### **5.3 Determination of maximal timolol maleate concentration in growth media that allow sustained J-HCET viability over 21 days (504 hours).**

#### **5.3.1 Introduction**

Preliminary studies were carried out to determine the maximal concentration of timolol maleate, in growth medium, that would not alter the J-HCET viability over a 21 day (504 hour) exposure. A range of concentrations were employed, to determine a toxicity dose response, assessed using resazurin reduction- glutathione equivalents and kenacid blue assays.

#### **5.3.2 Materials and Methods**

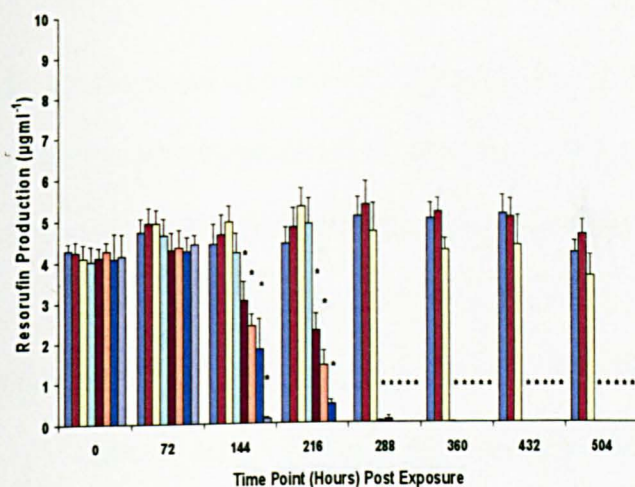
Immediately prior to use timolol maleate was completely dissolved in growth medium (section 2.1.7.1) (containing 1mM calcium chloride) to give concentrations of 0.17, 0.25, 0.36, 0.54, 0.79, 1.16 and 1.70mgml<sup>-1</sup>. Concentrations were made up in medium containing either the HKGS or HKGS-V2 supplements. J-HCET were seeded into the central 54 wells of eight 96 well plates at 3x10<sup>5</sup> cells ml<sup>-1</sup> (100µl). Phosphate buffered saline (PBS) (100µl) was applied to the outer 36 wells to prevent excessive evaporation of growth media. Plates were incubated for 72 hours at 37°C and 5% (v/v) CO<sub>2</sub> in air to allow cells to attach and attain confluence. One plate was assayed following the 72 hour growth period, providing a pre-exposure control. Medium was aspirated and the resazurin reduction (section 2.2.4.2), glutathione equivalents (section 2.2.4.4) and kenacid blue (section 2.2.4.1) assays performed.

The medium in the remaining plates was replaced with 200µl of medium containing the timolol maleate concentrations. For each concentration 6 replicate wells were employed. Plates were refed with fresh culture medium containing the appropriate timolol maleate concentration every 72 hours and incubated at 37°C and 5% CO<sub>2</sub> (v/v) in air. The resazurin reduction, glutathione equivalents and kenacid blue assays were performed every 72 hours over 504 hours.

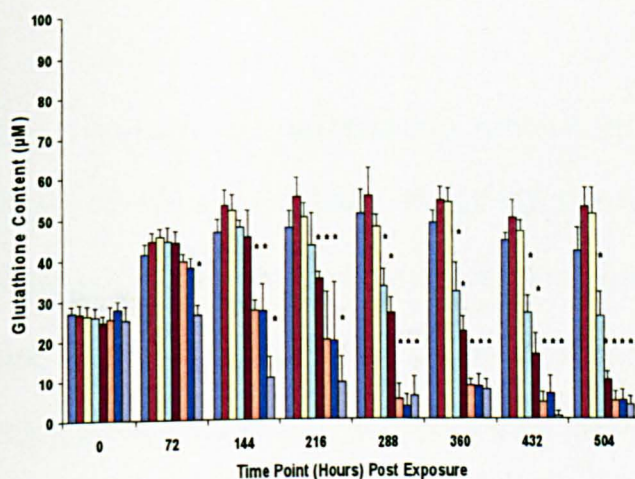
### **5.3.3 Results**

Only at 0.25mgml<sup>-1</sup> timolol and below was the culture activity (figures 5.10 and 5.13), GSH (figures 5.11 and 5.14) and total protein content (figures 5.12 and 5.15) unaffected, compared with untreated controls. Most concentrations caused a significant reduction in culture activity and GSH concentration at 144 hours post exposure whilst a concentration of 1.7mgml<sup>-1</sup> causes a significant GSH reduction at 72 hours. A similar pattern is observed regardless of supplement type employed i.e. HKGS or HKGS-V2

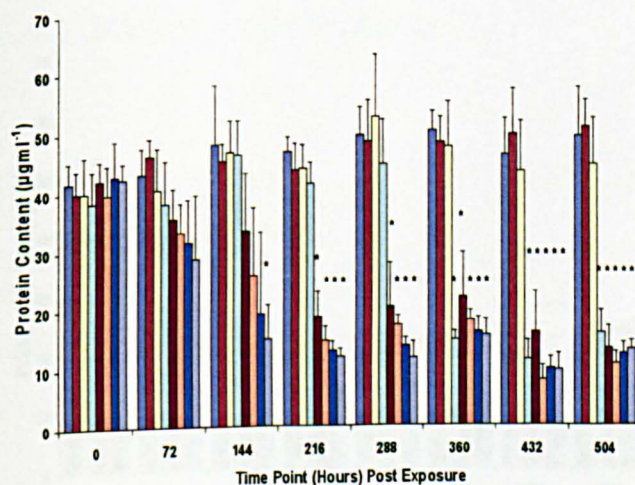




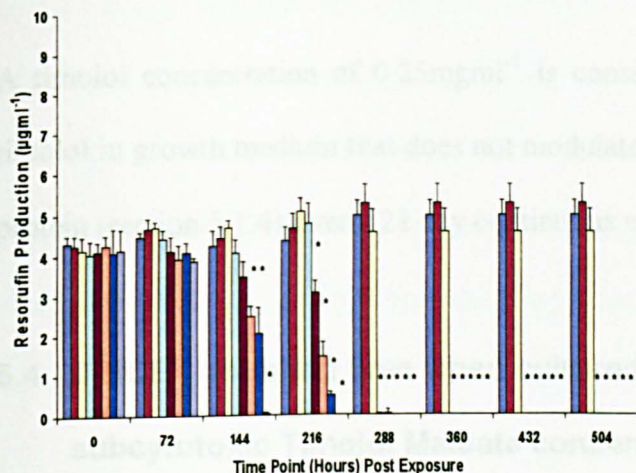
**Figure 5.10 :** Production of resorufin equivalents ( $\mu\text{gml}^{-1}$ ) of J-HCET monolayers cultured in HKGS supplemented media containing 0  $\text{mgml}^{-1}$  ■ 0.17  $\text{mgml}^{-1}$  ■ 0.25  $\text{mgml}^{-1}$  ■ 0.36  $\text{mgml}^{-1}$  ■ 0.54  $\text{mgml}^{-1}$  ■ 0.79  $\text{mgml}^{-1}$  ■ 1.16  $\text{mgml}^{-1}$  ■ and 1.70  $\text{mgml}^{-1}$  Timolol Maleate over 505 hours where \* denotes a significant ( $P < 0.05$ ) decrease in resorufin production compared to control cultures (mean+S.D.,  $n=3$ ).



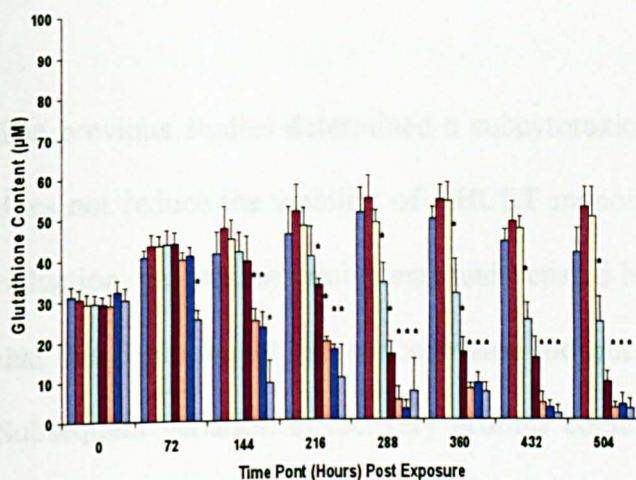
**Figure 5.11 :** Glutathione content ( $\mu\text{M}$ ) of J-HCET monolayers cultured in HKGS supplemented media containing 0  $\text{mgml}^{-1}$  ■ 0.17  $\text{mgml}^{-1}$  ■ 0.25  $\text{mgml}^{-1}$  ■ 0.36  $\text{mgml}^{-1}$  ■ 0.54  $\text{mgml}^{-1}$  ■ 0.79  $\text{mgml}^{-1}$  ■ 1.16  $\text{mgml}^{-1}$  ■ and 1.70  $\text{mgml}^{-1}$  Timolol Maleate over 505 hours where \* denotes a significant ( $P < 0.05$ ) decrease in glutathione content compared to control cultures (mean+S.D.,  $n=3$ ).



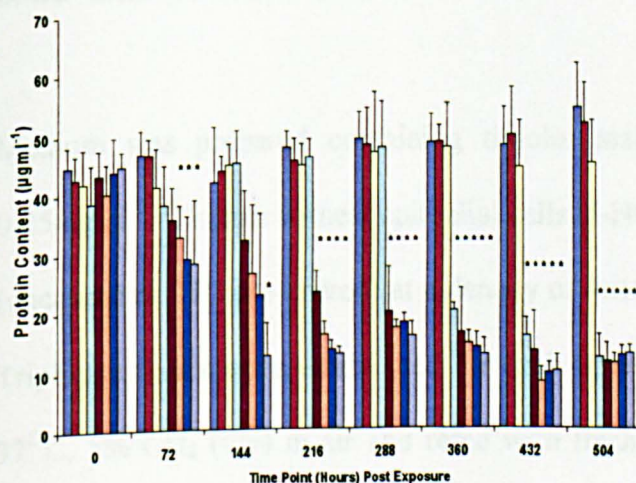
**Figure 5.12 :** Protein content ( $\mu\text{gml}^{-1}$ ) of J-HCET monolayers cultured in HKGS supplemented media containing 0  $\text{mgml}^{-1}$  ■ 0.17  $\text{mgml}^{-1}$  ■ 0.25  $\text{mgml}^{-1}$  ■ 0.36  $\text{mgml}^{-1}$  ■ 0.54  $\text{mgml}^{-1}$  ■ 0.79  $\text{mgml}^{-1}$  ■ 1.16  $\text{mgml}^{-1}$  ■ and 1.70  $\text{mgml}^{-1}$  Timolol Maleate over 505 hours where \* denotes a significant ( $P < 0.05$ ) decrease in protein content compared to control cultures (mean+S.D.,  $n=2$ ).



**Figure 5.13** : Production of resorufin equivalents ( $\mu\text{gml}^{-1}$ ) of J-HCET monolayers cultured in HKGS-V2 supplemented media containing  $0 \text{ mgml}^{-1}$ ,  $0.17 \text{ mgml}^{-1}$ ,  $0.25 \text{ mgml}^{-1}$ ,  $0.36 \text{ mgml}^{-1}$ ,  $0.54 \text{ mgml}^{-1}$ ,  $0.79 \text{ mgml}^{-1}$ ,  $1.16 \text{ mgml}^{-1}$ , and  $1.70 \text{ mgml}^{-1}$  Timolol Maleate over 505 hours where \* denotes a significant ( $P < 0.05$ ) decrease in resorufin production compared to control cultures (mean+S.D.,  $n=3$ ).



**Figure 5.14** : Glutathione content ( $\mu\text{M}$ ) of J-HCET monolayers cultured in HKGS-V2 supplemented media containing  $0 \text{ mgml}^{-1}$ ,  $0.17 \text{ mgml}^{-1}$ ,  $0.25 \text{ mgml}^{-1}$ ,  $0.36 \text{ mgml}^{-1}$ ,  $0.54 \text{ mgml}^{-1}$ ,  $0.79 \text{ mgml}^{-1}$ ,  $1.16 \text{ mgml}^{-1}$ , and  $1.70 \text{ mgml}^{-1}$  Timolol Maleate over 505 hours where \* denotes a significant ( $P < 0.05$ ) decrease in glutathione content compared to control cultures (mean+S.D.,  $n=3$ ).



**Figure 5.15** : Protein content ( $\mu\text{gml}^{-1}$ ) of J-HCET monolayers cultured in HKGS-V2 supplemented media containing  $0 \text{ mgml}^{-1}$ ,  $0.17 \text{ mgml}^{-1}$ ,  $0.25 \text{ mgml}^{-1}$ ,  $0.36 \text{ mgml}^{-1}$ ,  $0.54 \text{ mgml}^{-1}$ ,  $0.79 \text{ mgml}^{-1}$ ,  $1.16 \text{ mgml}^{-1}$ , and  $1.70 \text{ mgml}^{-1}$  Timolol Maleate over 505 hours where \* denotes a significant ( $P < 0.05$ ) decrease in protein content compared to control cultures (mean+S.D.,  $n=2$ ).



### **5.3.4 Conclusion**

A timolol concentration of  $0.25\text{mgml}^{-1}$  is considered the highest concentration of timolol in growth medium that does not modulate the cell activity, GSH level or total protein (section 5.1.4) over a 21 day continuous exposure.

## **5.4 J-HCET doubling time when cultured in growth media containing a subcytotoxic Timolol Maleate concentration**

### **5.4.1 Introduction**

The previous studies determined a subcytotoxic timolol maleate concentration that does not reduce the viability of J-HCET monolayers as measured by the resazurin reduction, glutathione equivalents and kenacid blue assays. It is essential to ensure that this  $\beta$  blocker at this concentration did not alter the population doubling time. Subsequent variation in recovery profiles could then be considered to be due to a depression or acceleration of J-HCET mitotic activity.

### **5.4.2 Materials and Methods**

Medium was prepared containing timolol maleate at a final concentration of  $0.25\text{mgml}^{-1}$ . Human corneal epithelial cells (J-HCET) were seeded into  $25\text{cm}^2$  flasks (uncoated or TIC pre-coated) at a density of  $4 \times 10^4$  cells per flask in 5ml of medium. Triplicate flasks were established for each time point. Cultures were maintained at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$  (v/v) in air and refed with fresh medium every 48 hours. J-HCET

were harvested (section 2.2.3.3) after 24 hours, and subsequently every 24 hours up to 120 hours and the cell number determined using a haemocytometer (section 2.2.3.2). After 120 hours of exposure the cells were re-seeded into appropriately labelled 25cm<sup>2</sup> flasks and cultured for a further 120 hours, with the cell number determined every 24 hours to generate a growth curve. This process was repeated over 504 hours. The population doubling time was calculated using the previous equation (section 3.1).

### **5.4.3 Results**

Timolol maleate (0.24mgml<sup>-1</sup>) exposure does not significantly alter the growth rate of J-HCET cultured over 504 hours utilising either HKGS or HKGS-V2 supplements (table 5.01). In addition, the recommended coating matrix for use with the HKGS-V2 supplements was not removed by the subcytotoxic concentrations as occurred with subcytotoxic surfactant concentrations (section 3.1).

**Table 5.01 :** Population doubling times of J-HCET cultured in HKGS or HKGS-V2 supplemented medium containing subcytotoxic timolol maleate concentrations (mean±S.D, n=3).

Supplement	Exposure	Doubling Time (Hours) Between Days			
		0 - 5	5 - 10	10 - 15	15 - 21
HKGS	None (Control)	22±1.3	21±0.5	21±0.1	21±0.1
	Timolol Maleate	22±0.9	21±0.3	20±0.3	21±0.1
HKGS-V2 <sup>m</sup>	None (Control)	21±0.5	20±0.4	21±0.8	20±0.9
	Timolol Maleate	21±0.2	20±0.4	21±0.5	21±0.2
HKGS-V2	None (Control)	30±5.6	30±0.5	28±0.1	29±0.3
	Timolol Maleate	31±0.8	29±0.5	27±1.4	28±1.1

### 5.5 Evaluation of the effects of chronic subcytotoxic timolol maleate exposure to a human corneal cell line

#### 5.5.1 Introduction

This study aims to investigate the chronic adverse effects of exogenous chemicals, using a J-HCET monolayer model. Timolol maleate has the potential to have adverse side effects on the corneal epithelium. Studies were conducted to determine the effects chronic timolol exposure had on the subsequent activity and glutathione content following an acute (60 second) Timolol exposure.

### 5.5.2 Materials and Methods

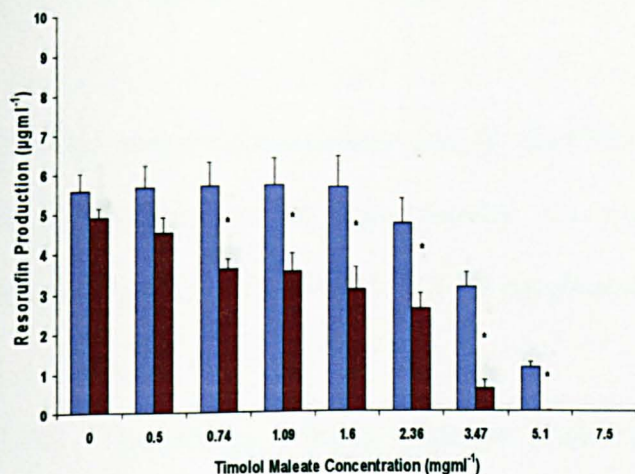
Culture flasks (80cm<sup>3</sup>) were seeded with  $5 \times 10^5$  of J-HCET cells and incubated for 24 hours at 37°C, 5% (v/v) CO<sub>2</sub> in air. Medium or medium containing timolol maleate (0.24mgml<sup>-1</sup>) was added to each flask where appropriate. Cultures were maintained at 37°C, 5% (v/v) CO<sub>2</sub> in air, re-fed with the appropriate medium every 72 hours over 432 hours (18 days) and subcultured once cells were 70-80% confluent. Cells were monitored on a daily basis at low magnification (x40) to ascertain the level of cell growth, cell morphology and to check for contamination. Following the 18 day exposure, J-HCET cells were seeded into the central 54 wells of two 96 well plates at  $3 \times 10^5$  cells ml<sup>-1</sup> (100µl). Phosphate buffered saline (100µl) was applied to the outer 36 wells to prevent excessive evaporation of growth medium. Plates were incubated for 72 hours at 37°C and 5% CO<sub>2</sub> (v/v) in air to allow the cells to attach and attain confluence. One plate was assayed following the 72 hour growth period, providing a pre-exposure control. Medium was aspirated and the resazurin reduction (section 2.2.4.2), glutathione equivalents (section 2.2.4.3) and kenacid blue (section 2.2.4.1) assays performed. Immediately prior to use, timolol maleate was completely dissolved in HBSS (containing 1mM calcium chloride) to create concentrations of 0.5, 0.74, 1.09, 1.6, 2.36, 3.47, 5.1 and 7.5mgml<sup>-1</sup>. The medium was aspirated from the remaining plates and 200µl of each of the timolol maleate concentrations or HBSS alone for 60 seconds. Wells were aspirated and the monolayers washed twice with 200µl of HBSS (60 seconds) containing 1mM calcium chloride. HBSS was aspirated and 200µl of cell culture medium (pre-warmed to 37°C) applied. Plates were incubated at 37°C and 5% CO<sub>2</sub> (v/v) in air for 48 hours. The resazurin



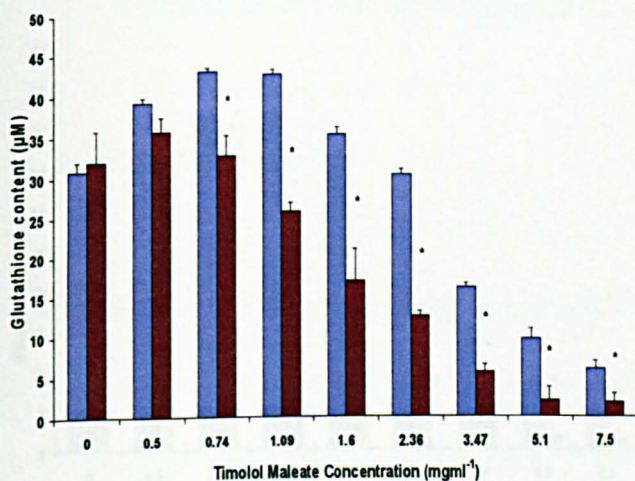
reduction, glutathione equivalents and kenacid blue assays were performed on the remaining plate.

### **5.5.3 Results**

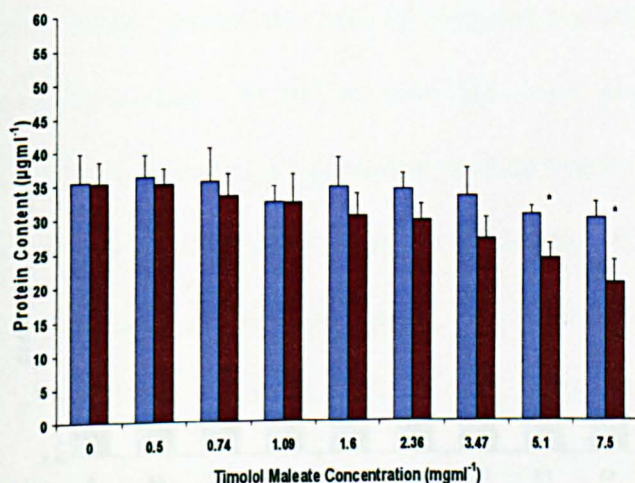
In J-HCET cultured in medium containing HKGS supplements pre-exposure to  $0.24\text{mgml}^{-1}$  timolol increased the toxic response of subsequent acute concentrations between  $0.74$  and  $5.1\text{mgml}^{-1}$  as indicated by a significant ( $P>0.05$ ) decrease in the production of resorufin compared to control cultures (figure 5.16). Additionally, the stimulation of GSH levels at timolol concentrations of between  $0.5$  and  $1.6\text{mgml}^{-1}$  was annulled (figure 5.17) with a dose dependent decline in GSH levels across the range of concentrations employed. Measurements of total culture protein demonstrate a significant reduction compared to control cultures at concentrations of  $5.1$  and  $7.5\text{mgml}^{-1}$  (figure 5.18). A comparable pattern of increased toxicity was observed in J-HECT cultured in HKGS-V2 supplemented medium as revealed by resorufin production (figure 5.19), glutathione levels (figure 5.20) and total protein content (figure 5.21).



**Figure 5.16 :** Resorufin production ( $\mu\text{gml}^{-1}$ ) of J-HCET monolayers cultured in HKGS supplemented medium ■ no pre-exposure or ■ Timolol ( $0.24\text{mgml}^{-1}$ ) 504 hour pre-exposure subsequently exposed to Timolol Maleate concentrations for 48 hours. Where \* denotes a significant ( $P<0.05$ ) decrease in resorufin compared to control (no pre-exposure) cultures at the same time point. (mean+S.D.,  $n=3$ ).

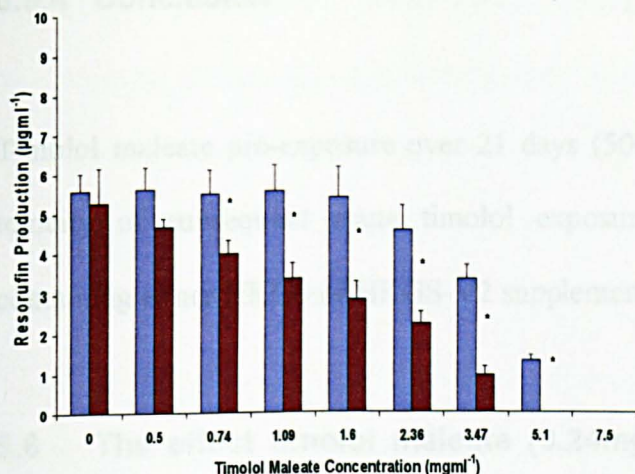


**Figure 5.17 :** Glutathione content ( $\mu\text{M}$ ) of J-HCET monolayers cultured in HKGS supplemented medium ■ no pre-exposure or ■ Timolol ( $0.24\text{mgml}^{-1}$ ) 504 hour pre-exposure subsequently exposed to Timolol Maleate concentrations for 48 hours. Where \* denotes a significant ( $P<0.05$ ) decrease in glutathione compared to control (no pre-exposure) cultures at the same time point. (mean+S.D.,  $n=3$ ).

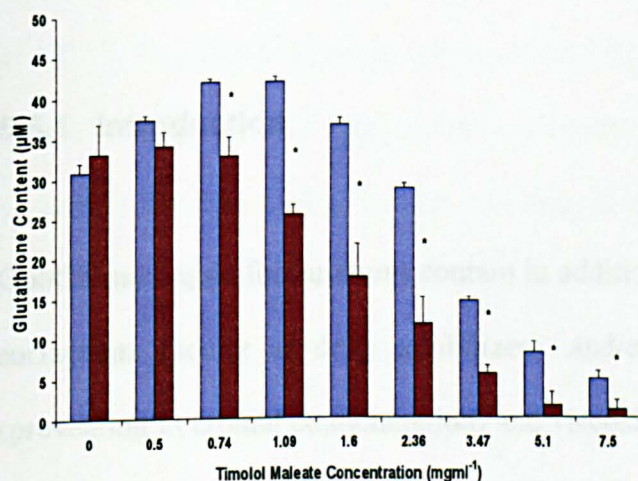


**Figure 5.18 :** Protein content ( $\mu\text{gml}^{-1}$ ) of J-HCET monolayers cultured in HKGS supplemented medium ■ no pre-exposure or ■ Timolol ( $0.24\text{mgml}^{-1}$ ) 504 hour pre-exposure subsequently exposed to Timolol Maleate concentrations for 48 hours. Where \* denotes a significant ( $P<0.05$ ) decrease in protein compared to control (no pre-exposure) cultures at the same time point. (mean+S.D.,  $n=3$ ).

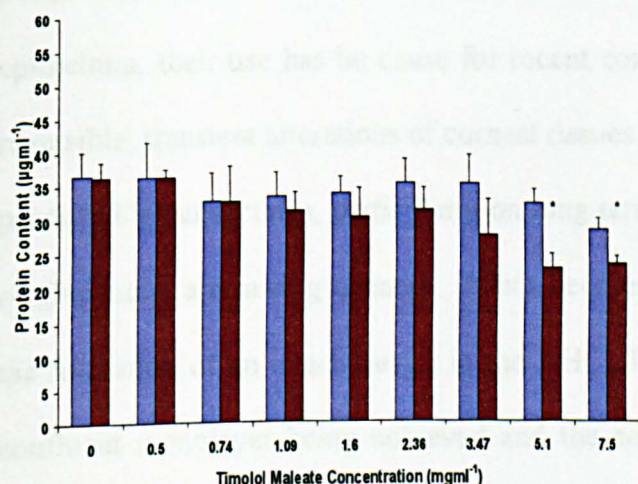




**Figure 5.19 :** Resorufin production ( $\mu\text{gml}^{-1}$ ) of J-HCET monolayers cultured in HKGS-V2 supplemented medium ■ no pre-exposure or ■ Timolol ( $0.24\text{mgml}^{-1}$ ) 504 hour pre-exposure subsequently exposed to Timolol Maleate concentrations for 48 hours. Where \* denotes a significant ( $P<0.05$ ) decrease in resorufin compared to control (no pre-exposure) cultures at the same time point. (mean+S.D.,  $n=3$ ).



**Figure 5.20 :** Glutathione content ( $\mu\text{M}$ ) of J-HCET monolayers cultured in HKGS-V2 supplemented medium ■ no pre-exposure or ■ Timolol ( $0.24\text{mgml}^{-1}$ ) 504 hour pre-exposure subsequently exposed to Timolol Maleate concentrations for 48 hours. Where \* denotes a significant ( $P<0.05$ ) decrease in glutathione compared to control (no pre-exposure) cultures at the same time point. (mean+S.D.,  $n=3$ ).



**Figure 5.21 :** Protein content ( $\mu\text{gml}^{-1}$ ) of J-HCET monolayers cultured in HKGS-V2 supplemented medium ■ no pre-exposure or ■ Timolol ( $0.24\text{mgml}^{-1}$ ) 504 hour pre-exposure subsequently exposed to Timolol Maleate concentrations for 48 hours. Where \* denotes a significant ( $P<0.05$ ) decrease in protein compared to control (no pre-exposure) cultures at the same time point. (mean+S.D.,  $n=3$ ).

#### **5.5.4 Conclusion**

Timolol maleate pre-exposure over 21 days (504 hours) significantly increased the toxicity of subsequent acute timolol exposure in J-HCET cultured in media containing either HKGS or HKGS-V2 supplements.

#### **5.6 The effect timolol maleate ( $0.24\text{mgml}^{-1}$ ) pre-exposure has upon barrier integrity when J-HCET monolayers are exposed to a clinically relevant concentration ( $2.5\text{mgml}^{-1}$ ) of timolol maleate**

##### **5.6.1 Introduction**

Ophthalmic liquid formulations contain in addition to drug and water, adjuvants: e.g. surfactants (acting as drug solubilizers, and/or spreading agents); preservatives (preventing microbial contamination) and viscosity increasing agents (prolonging the time of residence in the periocular area) (Chetoni *et al.*, 2003). Since ocular penetration enhancers act by inducing ultrastructural changes in the corneal epithelium, their use has been cause for recent concern (Chetoni *et al.*, 2003). While reversible, transient alterations of corneal tissues might be acceptable, ocular damage produced by surfactants, particularly on long term administration as in the treatment of glaucoma, are raising concern. It has been established (section 4.1 and 4.2) that the formation of an intact barrier in the J-HCET cell line is dependent upon both a confluent monolayer being achieved and the correct level of extracellular calcium chloride (1mM). Timolol maleate is known to have some calcium/sodium channel blocking activities (Osborne *et al.*, 2004) blunting the influx of calcium into stressed

cells (Melena *et al.*, 1999; Chidlow *et al.*, 2000). Therefore, studies were conducted to determine if chronic subcytotoxic timolol exposure affects the recovery of J-HCET barrier function subsequent to a clinically relevant acute timolol exposure.

### **5.6.2 Materials and Methods**

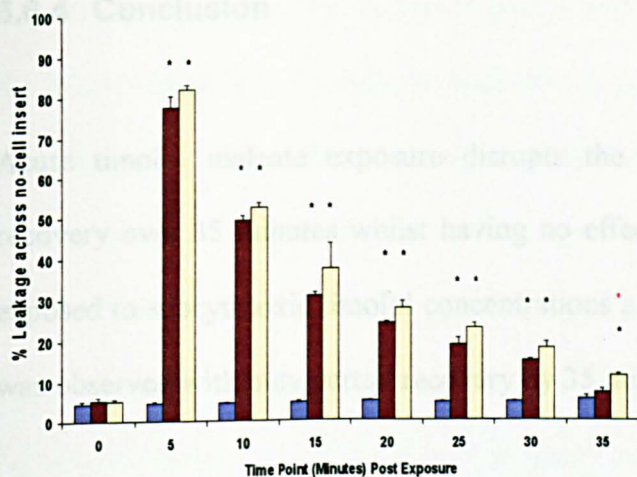
Culture flasks (80cm<sup>3</sup>) were seeded with 5x10<sup>5</sup> of J-HCET cells and incubated for 24 hours at 37°C, 5% (v/v) CO<sub>2</sub> in air. Medium or medium containing timolol maleate (0.24mgml<sup>-1</sup>) was added to each flask where appropriate. Cultures were maintained at 37°C, 5% (v/v) CO<sub>2</sub> in air, re-fed with the appropriate medium every 72 hours over 432 hours (18 days) and subcultured once cells were 70-80% confluent. Cells were monitored on a daily basis at low magnification (x10 objective) to ascertain the level of cell growth, cell morphology and to check for contamination. Following the 18 day exposure, J-HCET cells were harvested from the large culture flasks (section 2.2.3.3) and medium culture flasks (80cm<sup>3</sup>) were seeded with 5x10<sup>5</sup> of J-HCET cells and incubated for 24 hours at 37°C, 5% (v/v) CO<sub>2</sub> in air. Medium or medium containing timolol maleate (0.24mgml<sup>-1</sup>) was added to each flask where appropriate. Cultures were maintained at 37°C, 5% (v/v) CO<sub>2</sub> in air, re-fed with the appropriate medium every 72 hours over 432 hours (18 days) and subcultured once cells were 70-80% confluent. Cells were monitored on a daily basis at low magnification (x10 objective) to ascertain the level of cell growth, cell morphology and to check for contamination. Following the 18 day exposure, J-HCET cells were harvested from the culture flasks (section 2.2.3.3) and 500µl seeded into polycarbonate inserts at 5x10<sup>5</sup> cells ml<sup>-1</sup> suspended in the respective growth medium. Growth media (500µl) was added to the outer 16 wells to prevent excessive evaporation. Two inserts

received 500 $\mu$ l of medium alone and were referred to as the no-cell inserts. Inserts were placed into 24 well plates with an additional 500 $\mu$ l of corresponding medium in the wells and incubated at 37°C, 5% CO<sub>2</sub> (v/v) in air for 72 hours. Medium was aspirated from one plate and the combined fluorescein leakage/resazurin reduction (section 2.2.4.3) and total protein kenacid blue (section 2.2.4.1) assays performed. From the remaining plates medium was aspirated and 200 $\mu$ l of a 2.5mgml<sup>-1</sup> timolol maleate solution (in HBSS) applied for 60 seconds. Inserts were aspirated and the monolayers washed with 200 $\mu$ l of HBSS (2 x 60 seconds). HBSS was aspirated and 500 $\mu$ l of cell culture media (pre-warmed to 37°C) applied. The combined fluorescein leakage/resazurin reduction and kenacid blue assays were performed every 5 minutes over 35 minutes. One insert from each time point was set aside prior to kenacid blue exposure for immunohistochemical studies (section 5.7).

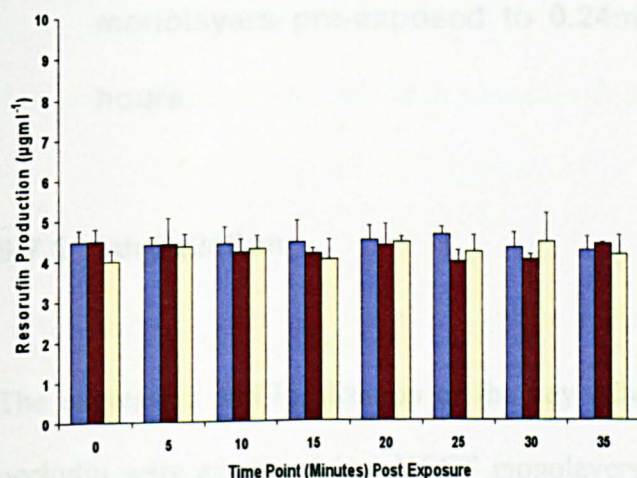
### **5.6.3 Results**

Acute (60 second) exposure to a timolol concentration of 2.5mgml<sup>-1</sup> significantly ( $P<0.05$ ) increased the level of fluorescein leakage across J-HCET monolayers 5 minutes post exposure compared to control cultures, with subsequent recovery at 35 minutes (figure 5.22). A similar effect was observed in J-HCET pre-exposed to timolol, with incomplete recovery observed at 35 minutes post exposure (figure 5.22). An acute (60 second) exposure to timolol (2.5mgml<sup>-1</sup>) did not significantly alter either the production of resorufin or total protein content of J-HCET monolayers cultured in media containing HKGS supplements either with or without prior subcytotoxic exposure (figures 5.23 and 5.34) over 35 minutes.

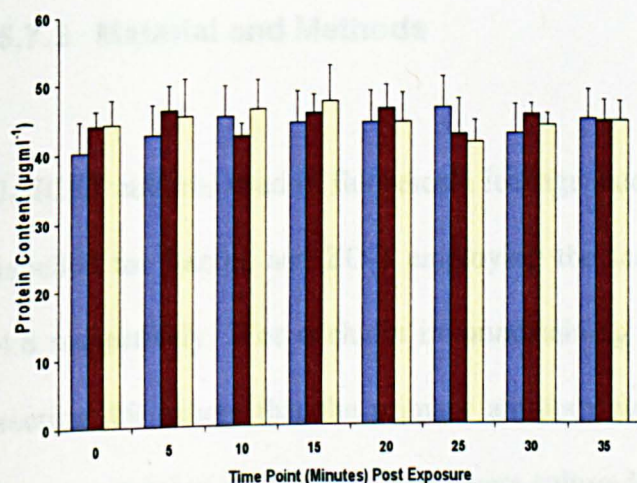




**Figure 5.22** : Fluorescein leakage across J-HCET monolayers cultured in ■ HKGS supplemented media (control), ■ exposed to 2.5mgml<sup>-1</sup> timolol maleate (60 seconds) or ■ cultured in media containing timolol maleate (0.24mgml<sup>-1</sup>) for 504 hours and subsequently exposed to a timolol concentration of 2.5mgml<sup>-1</sup> for 60 seconds. Where \* denotes a significant increase compared to control cultures and \* denotes a significant increase in leakage compared to non-pre-exposed cultures at a similar time point. (mean+S.D., n=3)



**Figure 5.23** : Resorufin production (µgml<sup>-1</sup>) in J-HCET monolayers cultured in ■ HKGS supplemented media (control), ■ exposed to 2.5mgml<sup>-1</sup> timolol maleate (60 seconds) or ■ cultured in media containing timolol maleate (0.24mgml<sup>-1</sup>) for 504 hours and subsequently exposed to a timolol concentration of 2.5mgml<sup>-1</sup> for 60 seconds. (mean+S.D., n=3)



**Figure 5.24** : Protein content (µgml<sup>-1</sup>) of J-HCET monolayers cultured in ■ HKGS supplemented media (control), ■ exposed to 2.5mgml<sup>-1</sup> timolol maleate (60 seconds) or ■ cultured in media containing timolol maleate (0.24mgml<sup>-1</sup>) for 504 hours and subsequently exposed to a timolol concentration of 2.5mgml<sup>-1</sup> for 60 seconds. (mean+S.D., n=3)

#### **5.6.4 Conclusion**

Acute timolol maleate exposure disrupts the barrier function, with subsequent recovery over 35 minutes whilst having no effect on cell activity. In cultures pre-exposed to subcytotoxic timolol concentrations a similar pattern of barrier disruption was observed with only partial recovery by 35 minutes post exposure.

### **5.7 Effects on adhesion molecule expression of an acute (60 second) timolol maleate ( $2.5\text{mgml}^{-1}$ ) exposure in J-HCET monolayers and monolayers pre-exposed to $0.24\text{mgml}^{-1}$ timolol maleate for 504 hours**

#### **5.7.1 Introduction**

The expression and localisation of the key adhesion molecules f-actin, ZO-1, and occludin were examined in J-HCET monolayers treated with timolol maleate, with samples set aside from section 5.6.

#### **5.7.2 Material and Methods**

J-HCET cultures used in fluorescein leakage studies (section 5.6) were retrieved and labelled for f-actin, and ZO-1 employing the same protocols as in sections 4.7 and 4.8 respectively. For occludin immunostaining a similar approach was taken as in section 4.9 except that the primary antibody was mouse anti-occludin (table 2.0). Immunostaining of J-HCET monolayers cultured in the keratinocyte medium (KGM)

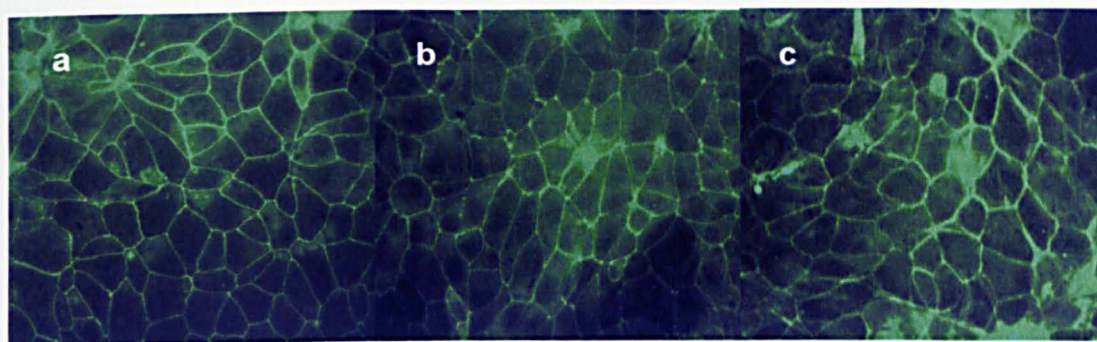
supplemented with animal derived products and calcium (100 $\mu$ M) routinely used in the ICCVAM/ECVAM validation trial (Strickland *et al.*, 2003) were employed as a control (figure 5.25). Slides were viewed under a Leica Confocal Microscope.

### **5.7.3 Results**

Immunohistochemistry controls were prepared as outlined in sections 4.7 through 4.9 to for f-actin, ZO-1, and occludin and examined microscopically confirming the efficacy of the staining protocol. The adhesion molecules f-actin, ZO-1 and occludin in J-HCET monolayers stained prior to acute timolol (2.5mgml<sup>-1</sup>) exposure demonstrate correct intracellular localisation (figure 5.25).



**Figure 5.25** : Images representative of (a) occludin, (c) ZO-1 and f-actin (d) expression in J-HCET monolayers cultured in HKGS supplemented medium (in the presence or absence of  $0.24\text{mgml}^{-1}$  timolol maleate) immediately prior to acute ( $2.5\text{mgml}^{-1}$ ) timolol maleate exposure. (magnification x400).



When the images of the adhesion molecules occludin, ZO-1 and f-actin (figure 5.25) prior to acute timolol exposure were interpreted in conjunction with the fluorescein leakage data (figure 5.22) the barrier function of the J-HCET monolayer was intact. Alterations in adhesion molecule expression (f-actin, ZO-1, and occludin) may not be attributed to the loss of cell confluency due to cell death as total culture protein measurements made at 5 minute intervals over the 35 minute period reveal no significant decrease compared to control cultures. This was further confirmed by propidium iodide staining of the J-HCET nuclei demonstrating the presence of an intact monolayer at all time points.

### **5.7.3.1 Effect of acute timolol exposure on f-actin, ZO-1 and occludin in non-pre-exposed J-HCET cultures**

The pre-exposure status (figure 5.25) and subsequently up to 10 minutes post timolol (2.5mg/ml-1) exposure f-actin expression was found at the periphery of the cell adopting the classical “chicken wire” appearance. At 15 minutes post exposure, f-actin expression begins to appear more concentrated in certain cells groups with bright fluorescence. This tends to mask the majority of the cells that are thus seen in paler “chicken wire” pattern. In certain cells there appears to be intense cytoplasmic labelling as if the timolol maleate exposure has stimulated f-actin production in certain cells (figure 5.26). Thus the peripheral location of the f-actin labelling is not lost during the 35 minutes post exposure. Immediately following acute timolol exposure and subsequently up to 10 minutes post exposure, ZO-1 immunostaining was completely lost from the cell-cell contact location. There is a focal point of labelling in the cell cytoplasm that could indicate that the ZO-1 is not lost but condensed into a vesicle within the cells. ZO-1 staining begins to appear at the periphery of abutting J-HCET 15 minutes post exposure with a few focal points still visible even at 35mins. Complete recover by 35 minutes post exposure in ZO-1 expression and localization (figure 5.26) is almost achieved but there is still some indications of beading of the expression along abutting cell membranes. Five minutes post acute timolol exposure and subsequently up to 15 minutes post exposure, occludin expression at the periphery of abutting J-HCET was completely disrupted. Occludin staining appears at the periphery of abutting J-HCET at 20 minutes post exposure with indications of recovery by 35 minutes post exposure in occludin expression and localisation (figure 5.26). However, this was not complete

and cytoplasmic staining was retained even at 35 minutes which render the appearance different from that of the control (c.f. figure 5.25b and figure 5.26 at 35 mins). Thus apart from the Occludin, where the level of labelling does appear to decline at 5 and 10 minutes the effect of timolol maleate at  $2.5\text{mgml}^{-1}$  results in relocation of the adhesion molecules rather than loss, which would facilitate rapid restitution of the barrier functioning.

#### **5.7.3.2 Effect of acute timolol exposure on f-actin, ZO-1 and occludin timolol pre-exposed J-HCET cultures**

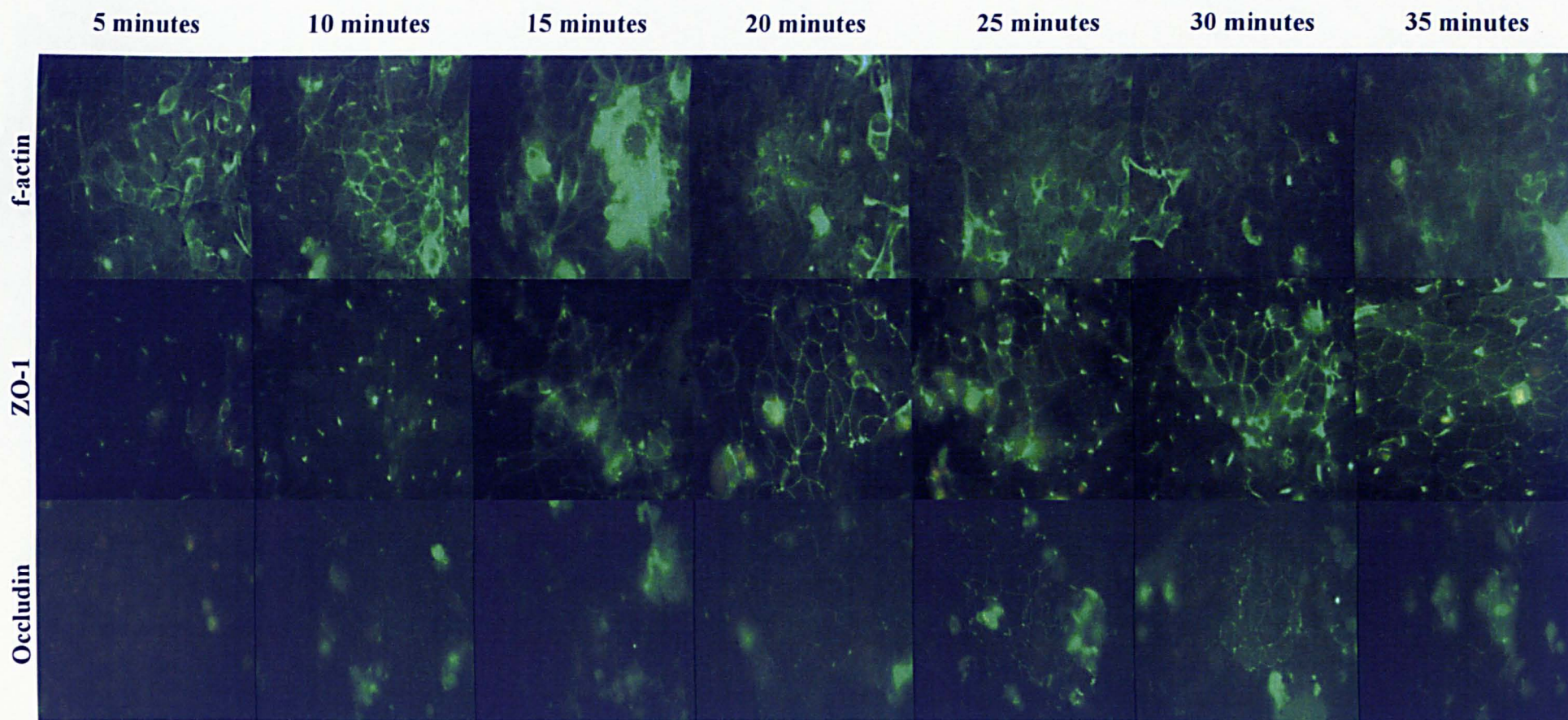
At pre-exposure and subsequently up to 10 minutes post timolol ( $2.5\text{mgml}^{-1}$ ) exposure f-actin expression was found at the periphery of the cell adopting the classical appearance of “chicken wire”. At 15 minutes post exposure, f-actin expression began to appear more cytoplasmic with the “chicken wire” pattern becoming indistinct with continued diffuse staining up to 35 minutes post exposure (figure 5.27). Immediately following acute timolol exposure and subsequently up to 10 minutes post exposure, ZO-1 immunostaining was completely disrupted with focal localisation in vesicles. ZO-1 staining appears at the periphery of abutting J-HCET 15 minutes post exposure which appears more rapid relocation than with the non-chronically exposed cells (figure 5.26 15mins verses 5.27 15 mins). Subsequently up to 35 minutes post exposure recovery in the ZO-1 expression and localization was the same as for the non-chronically exposed cells (c.f. figure 5.26 with figure 5.27). Five minutes post acute timolol exposure and subsequently up to 10 minutes post exposure, occludin expression at the periphery of abutting J-HCET was completely disrupted. Occludin staining appeared at the periphery of some of



the abutting J-HCET at 15 minutes post exposure with more complete recovery by 35 minutes post exposure in both expression and localisation (figure 5.27). This was again earlier than found in the non-chronically exposed timolol maleate cells (c.f. figure 5.26 with 5.27).

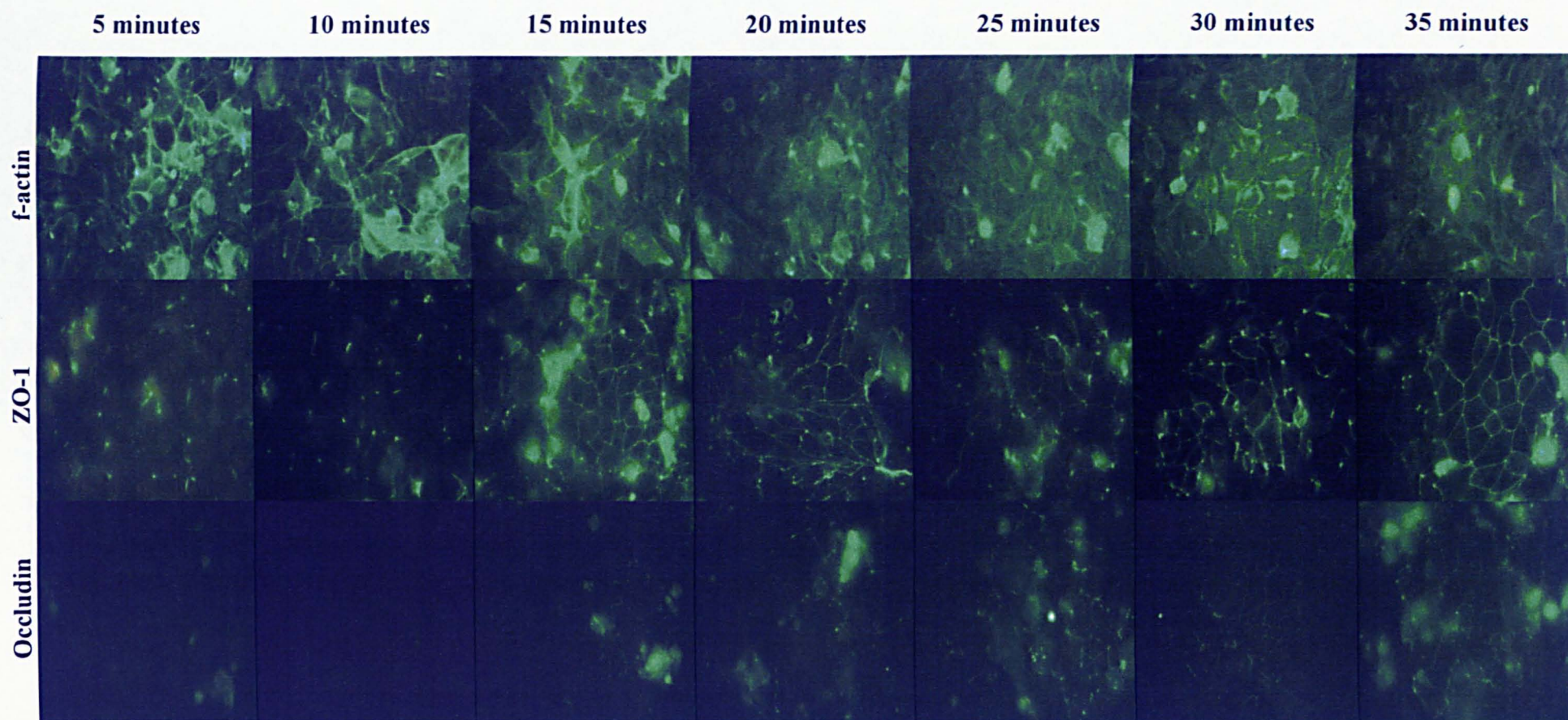
#### **5.7.4 Conclusion**

Acute timolol maleate exposure results in a relocation of the adhesion molecules ZO-1 and occludin from the cell-cell contact points within 5 minutes of application. This correlated with the reduction in the barrier function of J-HCET monolayers with respect of fluorescein leakage. Disrupting the location and/or expression of the adhesion molecules ZO-1 and occludin, essential for the formation of tight junctions, the barrier function is compromised. Over a period of 35 minutes, ZO-1 and occludin expression and localization had recovered sufficiently to restore the barrier function. When J-HCET were pre-exposed to a subcytotoxic timolol concentration similar effects were observed except ZO-1 and occludin were seen at the cells junctions after only 15 minutes and occludin expression was only partial restored by 35 minutes. This does indicate that the localisation of Occludin may be the more significant factor since there was a significant increase in fluorescein leakage at 35 minutes compared to the J-HCET exposed which had not been pre-exposed (figure 5.22).



**Figure 5.26 :** Expression and localisation of f-actin, ZO-1, and occludin over 35 minutes in J-HCET monolayers cultured in HKGS supplemented media exposed to Timolol maleate ( $2.5\text{mgml}^{-1}$ ) for 60 seconds.





**Figure 5.27** : Expression and localisation of f-actin, ZO-1, and occludin over 35 minutes in J-HCET monolayers cultured in HKGS supplemented media containing  $0.24\text{mgml}^{-1}$  timolol maleate subsequently exposed to  $2.5\text{mgml}^{-1}$  timolol for 60 seconds.

## **Chapter 6**

### ***General discussion***

# General discussion

## 6.1 Introduction

The study aimed to develop a simple human corneal epithelial cell (J-HCET) based model, based on the differential effects of chronic and acute exogenous chemical exposure, to predict potential *in vivo* eye irritation effects. In this model assess the change in cell activity and monolayer barrier integrity, was employed as a measure of acute to mild damage, after chronic/repeat exposure.

It has been established that the fluorescein leakage assay with MDCK cells (Tchao, 1988; Shaw *et al.*, 1990) can predict the irritant effects of chemicals (Brantom *et al.*, 1997) or cosmetic formulations (Cottin and Zanvit., 1997) that cause mild effects to corneal epithelium. In combination with the resazurin reduction assay (Clothier and Samson, 1996) an enhance prediction of chronic or repeat exposure effects has been produced for skin irritation (Clothier *et al.*, 1999). This was then transferred to human corneal epithelium *in vitro* (Clothier *et al.*, 2000).

Whilst measures of barrier function are important, observations concerning the expression and localisation of f-actin and its association with the key adhesion molecules, ZO-1, occludin and claudin-1 was also required since changes in these may not be detected as a change in cell activity but should be as leakage. The J-HCET monolayers (Araki-Sasaki *et al.*, 1995) were employed since the ability of cells to modulate toxic insults can differ even when they express similar adhesion molecules. Differential toxic responses were noted in the phototoxicity study

(Spielmann *et al.*, 1998; Clothier *et al.*, 1999) between the human corneal cells and human keratinocytes (Combes *et al.*, 1999).

Chronic exposure to subcytotoxic concentrations of four representative surfactants; BAK (cationic), SDS (anionic), CAPB (amphoteric) and T20 (non-ionic) and one clinically relevant pharmaceutical, timolol maleate, indicated that low concentration over a 21 day period do not affect the cell number, activity or barrier function, but may alter, marginally, the expression of the key adhesion molecules. Interactions between surfactants following chronic exposure to one surfactant, at subcytotoxic concentrations, modulated the subsequent J-HCET response to a subsequent acute exposure with the same or different surfactants. This revealed cross reactivity between the effects of different surfactants as described by Herlofson (1996) in the buccal cavity treated with CAPB or SDS. The aim of ascertaining if chronic exposure modulates the response to the same type of surfactant or to surfactants in general was achieved. Whilst standard surfactants were good indicators of the effects following chronic exposure, there were pharmaceuticals designed for repeat use in the eye e.g. timolol maleate in the treatment of glaucoma. Since timolol maleate was detoxified *in vivo* via conjugation with glutathione (GSH) (Megaw, 1984; Riley, 1990) the effects on glutathione levels could have consequences resulting following chronic exposure, allowing for the examination of a distinct intracellular detoxification pathway role in compromising the barrier function.



## 6.2 Development of the chronic exposure model

### 6.2.1 Evaluation of the J-HCET cell line

A number of validation studies; EC/HO (Balls *et al.*, 1995), CTFA (Gettings *et al.*, 1996), COLIPA (Brantom *et al.*, 1997), IRAG (Bradlaw *et al.*, 1997) and MHW/JCIA (Ohno *et al.*, 1999) have employed cell monolayers expressing adhesion junction *in vitro*, e.g. SIRC, HeLa, MDCK and CHL. Few of these studies have taken into account the specialised nature of the human corneal epithelium when considering toxicity endpoints or the associated adhesion molecules and detoxification pathways. According to the European Centre for the Validation of Alternative Methods (ECVAM) review of *in vitro* methods (Worth and Balls, 2002b), models need to match human *in vivo* expression of differentiated function with the relevant cell phenotypes. Two cell lines derived from human corneocytes have been used to model the corneal epithelium *in vitro*, the J-HCET (Araki-Sasaki *et al.*, 1995) and Gillette HCE-T (Kahn *et al.*, 1993; Ward *et al.*, 1997b) cell lines. Kahn *et al.*, (1993) infected primary human corneal explants with an Adeno 12-SV40 (Ad12-SV40) hybrid virus, resulting in a number human corneal epithelial cloned cell line with an extended life span. Although exhibiting an extended *in vitro* life span, the cell line employed was not continuous, and can only be utilised upto 20 generations (Kruszewski *et al.*, 1997), thereby limiting its availability and ultimate usefulness. Growth of this cell line upon a collagen membranes, at an air-liquid interface, promoted multilayering, more closely approximating corneal morphology observed *in situ* (Kahn *et al.*, 1993) retaining a variety of phenotypic characteristics (Ward *et al.*, 1997b). In contrast, Araki-Sasaki (*et al.*, 1995) isolated human corneal

epithelial cells which were subsequently transfected, with the SV40 virus to obtain a continuous growing cell line (J-HCET). This cell line was reported to grow for more than 400 generations exhibiting a cobblestone-like appearance similar to normal corneal epithelia in culture (Araki-Sasaki *et al*, 1995; Moore *et al*, 2005). The ability to progress past 20 generations was confirmed in this study, even in the presence of different media. When cultured at the air-liquid interface the transformed corneal cells differentiated in a multilayer fashion (Araki-Sasaki *et al*, 1995) similar to that reported for the HCE-T cell line (Ward *et al*, 1996). Although both the J-HCET and HCE-T cell lines meet the criteria set down in the ECVAM review of *in vitro* methods (Worth and Balls, 2002b) an important criterion for the selection of a cell line for use in a validated *in vitro* model was the availability and stability over time. The limited volume of material and the variability between cell cultures from different donor eyes makes it difficult to perform routine biochemical and physiological investigations on primary isolated human corneal epithelial cells (Bednarz *et al*, 2000). In terms of longevity, the J-HCET cell line is superior to the HCE-T line (Khan *et al*, 1993), due to its ability to undergo more than 400 generations before senescence.

An important consideration in the development of an *in vitro* model of chronic ocular toxicity was the selection of an appropriate growth medium. The growth of many cell types in culture requires serum in the medium (Castro-Munozledo and Hernandez-Quintero, 1997) as a source of hormones, cytokines, vitamins, attachment, spreading and growth factors (Tezel and Del Priore, 1998). The absence of these can have profound effects upon *in vitro* cultures (Tezel and Del Priore, 1998). With the identification, cloning and recombinant production of essential

growth factors several chemically defined, serum free media are now available commercially. The development of serum free cell culture medium has been stimulated (Taub, 1990; Bjare, 1992; Ranaldi *et al*, 2003; Fitzsimmons *et al*, 2004; Van der Valk *et al*, 2004) and increasing numbers of formulations for the culture of primary isolates and continuous cell lines are available (Taub, 1990; Bjare, 1992; Fitzsimmons *et al*, 2004) although serum free media are generally more cell type specific (Tezel and Del Priore, 1998). The collection of foetal bovine serum (FBS) is a controversial issue due to the possible distress suffered by the foetus prior to- and during harvesting (Van der Valk *et al*, 2003). With the upcoming implementation of the REACH program it has been estimated that the total worldwide consumption of FBS will significantly increase due to the expansion of *in vitro* toxicity testing (Van der Valk *et al*, 2003). In keeping with the principles of the Three R's of Russell and Burch, (1959) a chemically defined culture medium and supplement package (HKGS and HKGS-V2) manufactured by Cascade Biologics was selected. Most research into *in vitro* alternatives to the Draize eye test has been directed towards modelling acute toxicity. Consequently there was limited published data regarding the behaviour of the J-HCET cell line over exposure periods greater than 96 hours. In order to assess the potential use of the J-HCET (Araki-Sasaki *et al*, 1995) it was necessary to determine the phenotypic stability during prolonged culture on a synthetic membrane as the characteristic morphology of cell lines can be lost during prolonged culture (Engelmann and Bohnke, 1990; Albert *et al*, 1972; Turksen *et al*, 1989). The expression of functional and correctly expressed and located adhesion molecules including tight junctions, as well as the growth rate when cultured in the medium where the chosen endpoints.

### 6.2.2 Population doubling time of the J-HCET cell line

Many of the properties which can be used to differentiate a transfected cell from a non-transfected control were also observed when tumour cells were compared with their normal *in vivo* counterparts. Although the J-HCET cell line created by Araki-Sasaki *et al.*, (1995) has been employed in this study, due to its transformation, both the phenotypic stability and replicative capacity of the cell line may degenerate during prolonged *in vitro* culture (Freshney 1994). The degeneration of a cell line *in vitro* may be monitored by simply monitoring changes in the population doubling time. Population doubling time may be affected by senescence, and medium composition of different inhibitory or stimulatory factors, such as variations in nutrient concentration or hormonal effects in medium or toxic drugs (Freshney, 1994). The problem of primary and transfected cell lines retaining their *in vivo* characteristics when employed in *in vitro* toxicity studies has recently been addressed by Strickland *et al.*, (2003). They concluded that monitoring the population doubling time was essential to ensure that toxicity profiles were not modulated as a consequence of the long term usage of a specific batch of cells that were changing over time. In addition, when conducting chronic toxicity studies, alterations in the population doubling time may reveal subtle alterations in cell biochemistry not necessarily identified using the more common biological endpoints (e.g. resazurin reduction and neutral red uptake). From the data presented in a recent study by Araki-Sasaki *et al.*, (2000) the population doubling time of the J-HCET cell line, cultured in RPMI-1640 (Gibco BRL Inc., Grand Island, NY) supplemented with 10% FCS can be approximated to 48 hours. By investigating the population doubling time of the J-HCET cell line with the basic Epilife medium, but with two different

growth supplements, HKGS [bovine pituitary extract (0.2% v/v), bovine insulin ( $5\mu\text{gml}^{-1}$ ), hydrocortisone ( $0.18\mu\text{gml}^{-1}$ ), bovine transferrin ( $5\mu\text{gml}^{-1}$ ), human epidermal growth factor ( $0.2\text{ngml}^{-1}$ )] or HKGS-V2 [human recombinant insulin-like growth factor type-1, prostaglandin E-2, human recombinant epidermal growth factor, hydrocortisone and a proprietary plant extract at undisclosed concentrations] the effects of the change in medium was ascertained. The doubling time was extended by 50% as a result of HKGS-V2 supplement addition in the absence of the coating matrix. The increased doubling time observed with the HKGS-V2 supplement was in agreement with observations made by Cook *et al.*, (2003) concerning the population doubling time of primary keratinocytes, utilising the same combination of medium and supplements. Cook *et al.*, (2003) showed that the variation in doubling time between the HKGS and HKGS-V2 supplements was negated by pre-coating the tissue culture plasticware with a Type I Collagen attachment matrix. This was also true for the corneal cells. Interestingly, in the cornea, Type I Collagen forms the major component of the corneal stroma on which the epithelium sit (Suzuki *et al.*, 2003). Hence, the problem was not directly due to the effects of the plant growth supplements, rather than human/animal derived, on growth, but on the attachment of the cells when seeded in the respective media. Hence, when the recommended attachment factor was employed the population doubling time of J-HCET cultured in HKGS-V2 supplemented medium was the same as for the HKGS supplemented medium at around 24 hours. The attachment matrix had no effect on the doubling time of J-HCET cultured in HKGS supplemented medium. Interestingly, the population doubling time of the J-HCET cell line in medium containing either supplement was significantly shorter than that obtained by Araki-Sasaki *et al.*, (2000) when RPMI medium containing 10% FCS was employed.

These results suggest that the HKGS-V2 supplements manufactured by Cascade Biologics were limited in their ability to support cellular attachment to tissue culture plasticware without the use of an additional attachment matrix (Type I Collagen), or that the HKGS variant does contain some collagen. *In vivo*, corneal epithelial cells rest on a thin layer of extracellular matrix, called the basement membrane (BM), which plays an essential role in epithelial adhesion to the underlying Bowman's layer (Attachment of corneal epithelial cells to their underlying substratum is the process that forms the basis for establishment of cell migration, the assembly of complex adhesive structures, cell polarity and morphology (Steele *et al.*, 1997)). *In vitro*, the proliferation of anchorage dependent cells may only occur after adhesion to the culture surface. Cells adhere to a culture surface through a multi-step process. Initially the absorption of the attachment factors e.g. cold insoluble globulin (CIG) often secreted as fibronectin onto the culture surface, enable a) contact between the cell and the surface, b) attachment to the coated surface through interactions between multivalent heparin sulphate synthesized by the cell and CIG adsorbed onto the culture surface and c) finally spreading of the attached cells (Steele *et al.*, 1997). These processes were supported by the unevenly distributed negative surface charge of mammalian cells, and it was the density of the charges on the surfaces in contact, and not the polarity, that was the governing factor for adhesion. The variation in population doubling time between J-HCET cultured in HKGS or HKGS-V2 medium may be due to alterations of charge density on the cells or the absence of essential medium components as a result of the use of plant extracts.

It has been demonstrated that the initial attachment of cultured corneal epithelial cells to oxygen-containing synthetic polymers i.e. tissue culture polystyrene (TCPS)



was dependent upon the presence of exogenous vitronectin (Vn) found in the culture medium (Steele *et al.*, 1997). Vitronectin, also known as S-protein, serum spreading factor and epibolin, is a mixture of two monomeric glycoproteins (65 and 75kDa) present in blood and the extracellular matrix of many tissues. Vitronectin and fibronectin are the two major adhesive proteins in plasma and serum. Like many other adhesion molecules, vitronectin binds to cells through interaction of the Arg-Gly-Asp sequence in its cell binding domain with vitronectin-specific cell surface receptors (Steele *et al.*, 1997). When used as a thin coating on tissue culture surfaces, vitronectin is useful to promote cell attachment, spreading, proliferation, and differentiation. The largest proportion of vitronectin in cell culture medium comes from foetal calf serum (FCS). The defined medium and supplement package employed did not contain the exogenous animal serum. As an alternative, bovine pituitary extract (BPE) forms a vital component of the HKGS supplements. Asami *et al.*, (1984), Mattinger *et al.*, (2002), Cook *et al.*, (2003), have demonstrated that bovine pituitary extract may act as an alternative to serum for the *in vitro* culture of epithelial cells and may provide a source of the required attachment proteins. The use of Type I Collagen attachment factor returns the population doubling time of J-HCET to “normal” but still raises the question as to the way this could have affected responses to subsequent cytotoxic/irritant insults.

### **6.2.3 Acquisition of the polarised cell phenotype**

The European cosmetics industry has had a long term interest in the development, validation and use of non-animal alternatives to assess the safety of cosmetic ingredients and formulations and to set “safe” human exposure levels, either as stand

alone methods or part of a tiered testing strategy (Brantom *et al.*, 1997). The fluorescein leakage assay for integrity of the epithelial barrier function was evaluated in the COLIPA/EU study of coded cosmetic ingredients and formulations (Brantom *et al.*, 1997). This study was undertaken following the EC/OH study (Balls *et al.*, 1995). The EC/HO study was designed to identify those *in vitro* assays that were capable of predicting eye irritation, correlated to the Draize scores for chemicals with “good” *in vivo* data taken from the ECETOC list (ECETOC Technical Report 48 (1) 1992). Brantom *et al.*, (1997) demonstrated that there was a good correlation between *in vivo* irritancy classifications, of the restricted cosmetic ingredients list of test agents, and those determined using the fluorescein leakage assay. However, for an alternative method to be considered reliable, it must demonstrate both interlaboratory and intralaboratory reproducibility (Curren *et al.*, 1995; Balls and Karcher, 1995). Brantom *et al.*, (1999) evaluated the performance of the fluorescein leakage assay and concluded that although the fluorescein leakage assay demonstrated good interlaboratory reproducibility between the two investigating laboratories and a reasonable statistical fit within its prediction model, there was insufficient data to assess its reliability. In addition, the fluorescein leakage test was incapable of discriminating between MMAS scores of between 30 and 110 (Brantom *et al.*, 1997) and was, therefore, more suitable for distinguishing between non-irritants or moderate irritants, i.e. between R34 and non classified (Balls and Clothier., 1992). With the fixed dose approach distinction of R36 from R41 classified chemicals has been possible (Clothier *et al.*, 1994).

Monolayers of epithelial cell lines e.g. MDCK, J-HCET and HCE-T maintain low permeability through expression of tight junctions between the cells, and this

function was very important to avoid cellular damage after the ocular introduction of a xenobiotic (Zanvit *et al.*, 1999). Such tight junctions are expressed when the epithelial monolayers were grown on permeable supports (Cottin and Zanvit, 1997; Ward *et al.*, 1997b; Moore *et al.*, 2005) and were thus impermeable to most chemicals, including the non-toxic dye sodium fluorescein. Unlike many alternatives to the Draize test for predicting eye irritation from acute xenobiotic exposure, the fluorescein leakage assay specifically includes damage to the epithelial barrier function, an important first stage in the generation of ocular irritancy (Cottin and Zanvit, 1997). The formation of an effective barrier, *in vitro* depends upon formation of a confluent layer of cells. Previous studies with MDCK cells (Clothier and Samson 1996) have demonstrated that if the original cell seeding density was too high, then adherence to parts of the culture surface was compromised and that the small areas devoid of cells result in a high rate of fluorescein leakage. Barrier function studies conducted using the J-HCET cell line confirmed this observation, namely that J-HCET need to “grow” to confluency to ensure a fully formed barrier *in vitro* regardless of the growth supplements employed (e.g. HKGS or HKGS-V2). The establishment of a confluent monolayer was not sufficient for the formation of a barrier. The expression and correct localisation of the components of the tight junction, i.e. f-actin, ZO-1 and occludin were dependent on the medium calcium concentrations (Kruszewski *et al.*, 1997; Ward *et al.*, 1997b; Moore *et al.*, 2005). The use of defined media and supplement packages in this study permits exacting control over the calcium concentration in the cell culture medium. The degree of fluorescein leakage across J-HCET monolayer demonstrated that a calcium concentration of between 0.5mM and 1mM is required to form an effective barrier to the passage of fluorescein, similar to the 1mM specified with the Gillette human

corneal cells culture method (Khan *et al.*, 1993; Kruszewski *et al.*, 1997; Ward *et al.*, 1997b). In the absence of calcium, the J-HCET continue to grow but lack the typical “cobblestone” appearance described by Araki-Sasaki *et al.*, (1995) with space between neighbouring cells. Total cell protein measurements conducted in this study confirm that the alteration in fluorescein flux across the J-HCET monolayer was due to modifications of the adhesion molecules influenced by calcium concentration (Moore *et al.*, 2005), rather than areas of the culture surface being devoid of cells. In addition the plant derived supplements do not significantly alter the calcium concentration required to form an effective barrier. The attainment of the leakage of 6% or less, the level routinely accepted as indicative of the fully functional barrier with MDCK cells (Clothier and Samson, 1996) also applied to predict confluence with the J-HCET cells (Moore *et al.*, 2005), was confirmed, and was half that attainable with the RPMI medium with 10% FCS (Clothier, personal communication). TEM images clearly demonstrated the presence of pinocytotic vesicles at all faces of the corneal cells in monolayer. These may be responsible for the transport of material from the apical to the basolateral domain through the cell, thereby bypassing the tight junctions, and accounting for the barrier function *in vitro* retaining some leakage, even if well below 6%.

In addition to the cell seeding density, medium supplements and the calcium concentration, the choice of culture insert (providing a two chamber system) can affect responses (Ward *et al.*, 1997a). Tissue culture inserts are commercially available, with membrane growth surfaces manufactured from a variety of materials, with different pore sizes and pore densities. Experiments with MDCK cells revealed

that the composition of the insert could affect the apparent leakage characteristics (Ward *et al.*, 1997a). This led to research evaluating the predictivity and sensitivity of the fluorescein leakage assay using different insert types with their human corneal epithelial cell line (Wilkinson *et al.*, 2005). Chemical incompatibilities vary with different membrane material types (Ward *et al.*, 1997a; Cottin and Zanvit, 1997). For example, it has been demonstrated that acetic acid, the positive control within the standard fluorescein leakage protocol (Shaw *et al.*, 1990) utilising Anopore membranes, is incompatible with the Millicell-HA membrane (Cottin and Zanvit, 1997) where exposure to acetic acid led to the destruction of the insert material. Manufactures of inserts, utilised in the fluorescein leakage assay, have issued warnings of membrane incompatibilities, since these membrane are mainly intended for filtration purposes. Membrane destruction as a consequence of chemical incompatibility was not the only problem. Membrane adsorption or chemical binding effects were not always known or reported. Since the majority of published fluorescein leakage assay protocols (Tchao, 1988; Shaw *et al.*, 1990; Clothier and Samson, 1996; Moore *et al.*, 2005) entail an acute (minutes) exposure to high concentrations ( $\text{mgml}^{-1}$ ) of test material, differential binding could alter chemical exposure time to the cultures significantly (Ward *et al.*, 1997a). Ward *et al.*, (1997a) reported that cationic surfactants appeared to preferentially adsorb to particular types of membrane material and cause sodium fluorescein to bind to the membrane, giving false negatives, where the degree of leakage no longer correlates with the degree of damage to the monolayer. Chemical binding may occur when using the Draize test, binding to the corneal epithelium or tear film, so the differential binding to various membrane materials could be significant when *in vitro/in vivo* comparisons were made (Ward *et al.*, 1997a). In addition, the selection of a growth support has been

demonstrated to influence the growth and differentiation of morphological and biochemical characteristics of the MDCK cell line commonly employed in permeability studies (Cook *et al.*, 1989; Butor and Davoust, 1992).

Although the growth supplement employed (HKGS or HKGS-V2) can alter the J-HCET population doubling time, the development of an intact barrier was unaffected, even when the collagen 1 coating was omitted. In order to optimise the fluorescein leakage assay for use with the J-HCET cell line and medium package, the use of an attachment factor was omitted when inserts were used due to potential problems associated with attachment matrix degradation, binding to and penetration of fluorescein through the underlying matrix. Interestingly and in contrast to the cells grown directly on the culture plates, the development of an intact barrier was identical for both HKGS and HKHS-V2 supplemented medium. To understand how J-HCET cultured in HKGS-V2 supplemented medium were able to attach to the polycarbonate membrane without the use of the recommended attachment matrix transmission electron microscopy of cells on the insert was employed. The J-HCET cells project processes into the pores of the insert material, thereby anchoring the monolayer to the polycarbonate membrane, as seen with MDCK cells (Cottin and Zanvit, 1997). The *in vitro* use of the combined fluorescein leakage-resazurin reduction assay was considered a relevant mechanistic endpoint for the irritancy potential of cosmetic ingredients and formulations (Clothier *et al.*, 1995). An advantage of using the fluorescein leakage assay over transepithelial resistance (TER), to examine barrier function of epithelial sheets, was that it was dependent on the correct expression of the adhesion components of the tight junction *in situ*, and not the additional transportation of ions through the cells.



#### 6.2.4 Morphological analysis of components of the tight junction

Among the various intercellular junctions the tight junctions (TJ's), originally defined and identified as zonulae occludens (ZO) of simple epithelial and endothelial (Langbein *et al.*, 2002) are commonly regarded as well understood in terms of structure and function. They are characteristically recognised in electron microscopy as small subapical regions of direct contact between the plasma membranes of abutting cells, with very limited intermembraneous material or extracellular gaps and are often referred to as “sites of fusion” or “kissing points” (Langbein *et al.*, 2002). Recent studies reveal that the TJ complex includes both transmembrane proteins e.g. claudin or occludin and membrane associated proteins e.g. zonula occludins-1 (ZO-1), ZO-2 and ZO-3 (Balda and Matter, 2000; Tsukita *et al.*, 2001; Langbein *et al.*, 2002; Ban *et al.*, 2003). Occludin was the first transmembrane protein, of tight junctions, to be identified (Furuse *et al.*, 1993; Balda and Matter 2000). Occludin is a 60 – 65kDa protein with four transmembrane domains (Furuse *et al.*, 1993; Balda and Matter 2000; Ban *et al.*, 2003) with both N- and C- termini oriented into the cytoplasm (Balda and Matter 2000). Occludin appears to be the most important and relatively well characterized transmembrane protein of the tight junction (Kale *et al.*, 2003) with the C-terminal interacting *in vitro* with several intracellular scaffolding proteins including ZO-1, ZO-2, ZO-3 (Furuse *et al.*, 1993; Balda and Matter 2000; Ban *et al.*, 2003). Claudins were more recently discovered and are a family of tight junction proteins of 22-24kDa (Table 6.01). Claudin-1 and -2 were originally identified because they cofractionated with occludin from sonicated junctional fractions (Balda and Matter, 2003). However, there was no evidence for a direct interaction between occludin and claudin, and claudins do not copurify with occludin

from detergent-solubilised membranes. In contrast to occludin, individual claudins are generally expressed in only a restricted number of specific cell types – suggesting that they are associated with tissue specific functions. Thus in normal TJ's either occludin or claudin occurs.

**Table 6.0x :** Characteristic features of different claudins.

Claudin	Distinctive Characteristics
1	Present in high-resistance epithelial (e.g. cornea) and absent in leaky epithelia (e.g. proximal tubule)
2	Present in leaky epithelia (e.g. proximal tubule) and absent in tight epithelia (e.g. cornea)
3	Present in the tighter segments of the nephron
4	Its expression decreases paracellular conductance through a selective decrease in sodium permeability
5	Constitutes TJ strands in endothelial cells
6	Present in embryonic epithelia, overexpression generates defective epidermal permeability barrier
7	Down regulated in head and neck squamous cell carcinomas
8	Present in the tighter segments of the nephron

The effects of variable calcium concentration on the adhesion molecules of the J-HCET monolayer tight junctions were clearly revealed in the immunohistochemistry of the adhesion molecules confirming what was shown by Moore *et al.*, (2005) with a different keratinocyte medium. During morphogenesis, mechanical forces generated by the dynamic rearrangements of cell-cell contacts and the cytoskeleton modulate changes in cell shape, motility and function (Jamora and Fuch, 2002). For the establishment of an intact barrier, abutting J-HCET must be intimately associated through both adherens and tight junctions. These junctions are ultimately anchored via scaffolding proteins interacting with the f-actin cytoskeleton whose expression and correct localisation is known to be calcium dependent (Jamora and Fuch, 2002). The location of f-actin to the periphery of the J-HCET was confirmed to be calcium

concentration dependent, for at low calcium concentrations ( $\leq 60\mu\text{M}$ ) the f-actin was diffuse. As the calcium concentration is increased to 1mM the f-actin relocates to the periphery. Once f-actin had formed the internal cytoskeletal network, other components of the tight junction i.e. ZO-1, occludin and claudin-1 were correctly localised and interacted. The location of the ZO-1 and occludin to the cell membrane was confirmed to be equally calcium ion concentration dependent, in both the media, and the comparable effects of calcium when the cells were grown on the polycarbonate inserts and the fluorescein leakage measured also confirmed the calcium dependence of the formation of the adhesion molecules (Cereijido *et al.*, 1980; Meldolesi *et al.*, 2004). The expression of claudin-1 was found to be equally calcium ion concentration dependent. However, unlike f-actin, ZO-1 and occludin, the expression of claudin-1 to the periphery of abutting J-HCET was dependent upon multilayering. Yi *et al.*, (2000) demonstrated claudin-1 specific immunostaining in the wing and basal layers of rat corneal epithelium i.e. claudin-1 was only located in areas of stratification. Using RT-PCR, Yi *et al.*, (2000) also demonstrated the presence of claudin-1, -2, -3, -9, -14, and -15 transcripts suggesting the expression of these in the J-HCET cell line. However, no data was obtained in the study of Yi *et al.*, (2000) regarding the intracellular localisation of these claudin members *in vitro*. It is clear from the TEM photomicrographs that the areas of stratification upon the polycarbonate insert were limited and so the role that claudin-1 plays in this monolayer model, with regard to barrier function was equivocal. The physical barriers that epithelial sheets establish to the passage of molecules are highly selective, and that this selectivity is dependent upon their physiological location. The corneal epithelium for example was known to express claudin-1 (Furuse *et al.*, 1998; Yi *et al.*, 2000) and extremely tight junctions *in vivo*. However, *in vitro*, in the

monolayer model developed during this study, claudin-1 expression and localisation was limited. This leads to the hypothesis that occludin was able to form the backbone of a tight junction complex but for added functionality the expression of specific claudins were required in other areas of the epithelium. A calcium concentration of between 500 $\mu$ M and 1mM was required for the formation of an intact barrier to the passage of sodium fluorescein; this was in contrast to the level routinely employed by Clothier and Samson (1996) of 100 $\mu$ M with the MDCK cell line was employed, but the MDCK culture medium contained bovine serum that contained calcium ions, so the exact calcium concentration was unknown. Similar effects on adhesion molecules were observed regardless of supplement type employed. Hence the f-actin interactions with other components of the tight junction (Anderson *et al.*, 1993; Portiz *et al.*, 2004) occurred in both media even though the f-actin cytoskeleton was highly dynamic (Hall, 1994).

## **6.3 Application of the J-HCET culture model to the test compounds**

### **6.3.1 Chronic and acute toxicity of surfactants**

Surfactants are organic compounds which are broadly classified into four categories; anionic, cationic, amphoteric and non-ionic, each exhibiting unique properties and applications. Surfactants are ubiquitous within our daily environment, being significant active components in both household products, personal care products, cosmetics, pharmaceuticals and endogenously in the human liver. Over a lifetime our eyes will be regularly exposed inadvertently, through personal care products, or deliberately, through the use of ophthalmic pharmaceuticals to surfactants. Being

an important category of ingredient in cosmetics (Vives *et al.*, 1997), their potential capacity to cause ocular irritation and at what concentration is required information in accordance with duty of care legislation. Surfactants are indispensable as solubilising agents in the isolation, purification, and reconstitution or crystallisation of membrane proteins (Kragh-Hansen *et al.*, 1998). Furthermore, at lower non-solubilising concentrations (i.e. below the Critical Micelle Concentration; CMC), these compounds are known to permeabilise and perturb membrane structure and function (Kragh-Hansen *et al.*, 1998). The toxic effects of surfactants are thought to be related to the ionic nature and the amount of free surfactant present, dependent on the total concentration and the CMC in the conditions under which the cells are exposed (Maurer *et al.*, 1997; Vives *et al.*, 1997). The proportion of surfactant molecules present at the plasma membrane or as micelles in the bulk of the liquid depends on the surfactant concentration. At low concentrations (**A**; Figure 6.01), surfactants will be monomeric and thus target the charged cell surface particularly if they are ionic. As the surfactant becomes more concentrated, i.e. molecules increasing in number micelles begin to be formed (**B**) and the number of monomeric molecules decline. Thus the number of surfactant molecules available to bind to the cell membrane decreases. At a specific concentration (dependent upon temperature, pressure, ionic concentration of other salts and liquid phase) the surfactant will be predominantly arrange as micelles but with few monomeric molecules (**C**), such that the plasma membrane can now become completely loaded with surfactant.

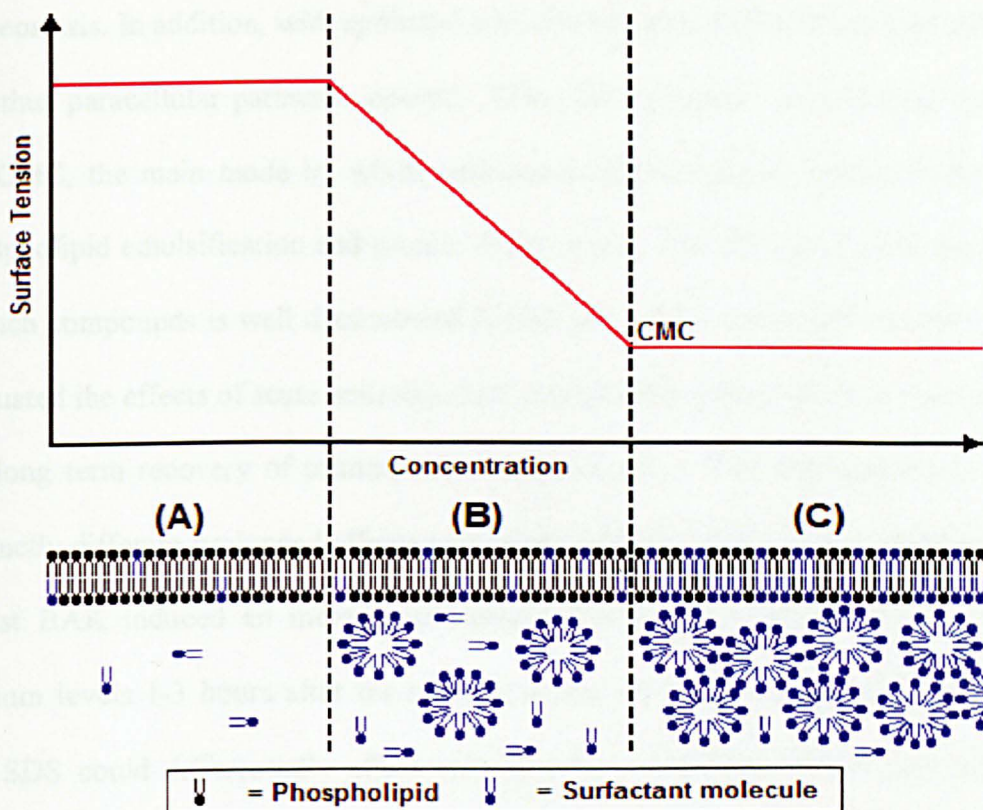
**Figure 6.01** : Physiochemical data concerning representative critical micelle concentrations of the test surfactants in growth medium (adapted from Ward *et al.*, 1998).

Surfactant	CMC (mM)
Sodium dodecyl sulphate	6.2 - 6.8
Tween 20	0.07 - 0.1
Cocamidopropylbetaine	0.2 - 0.24
Benzalkonium chloride	3.2 - 3.7

Thus the toxicity of a surfactant depends upon its CMC and the avidity with which it binds to the cell membrane, which is dependent in part on its charge. Thus the manifest toxicity is due to a set of interactions and the environment in which the surfactant is presented.

**Figure 6.02 :** A generic graph of surface tension versus the log of surfactant concentration.





Surfactants have the ability to induce phospholipid emulsification contributing to the cellular damage, which can result in cytolysis, the release of proteins, lysosomal and cytoplasmic enzymes and inflammatory mediators (Lee *et al.*, 2000). The sequence of events when membranes are exposed to increasing concentrations of surfactant is: increased permeability; lysis; solubilisation of lipid-protein-detergent associations; the appearance of lipid-detergent mixed micelles and solubilised protein; the appearance of detergent-only micelles or extensive denaturation and total solubilisation of proteins (Coleman, 1974; Vives *et al.*, 1997). Surfactants can thus induce cellular damage in a number of ways. At low concentrations ( $< \text{CMC}$ ), surfactant monomers incorporate into the plasma membrane, as the concentration increases pores are formed within the plasma membrane allowing the uncontrolled passage of molecules between the cytosol and the external environment, impairing

homeostasis. In addition, with epithelial cells the adhesion molecules can be affected and thus paracellular pathways opened. Once the surfactant concentration reaches the CMC, the main mode by which surfactants induce cellular damage is through phospholipid emulsification and protein denaturation. The effects of acute exposure to such compounds is well documented (ECETOC, 1997). Grant and Acosta (1996) evaluated the effects of acute benzalkonium chloride and sodium dodecyl sulphate on the long term recovery of primary rabbit corneal cells. The two surfactants have distinctly different prolonged effects on corneal epithelial cells. It was observed that whilst BAK induced an increase in intracellular calcium levels, SDS decreased calcium levels 1-3 hours after the removal of the surfactants suggesting that BAK and SDS could differentially affect cellular recovery *in vitro* (Grant and Acosta, 1996). However, the effects of chronic surfactant exposure on the corneal epithelium remain largely unknown, and need to be further examined (Clothier and Samson, 1996). Interactions between surfactants (exposure to SDS alleviates the effect of BAK and SDS) have been demonstrated to occur *in vivo* in skin (Grant and Acosta, 1996; McFadden *et al.*, 2001) although that this occurs at the ocular surface remains to be shown.

### **6.3.2 Rationale for the selection of a 21 day chronic-, and 60 second acute- exposure period**

A chronic subcytotoxic exposure period of 21 days was selected, as it has been demonstrated that prolonged chemical exposure allows both morphological and biochemical alterations to develop *in vitro* without loss of cell viability (Clemenson *et al.*, 1996; Clothier and Samson, 1996; Clothier *et al.*, 1997). In addition, when utilising the Draize test, the total effects due to initial exposure and the subsequent 21 days post inoculation are taken (Draize *et al.*, 1994). In one of the prescribed timolol preparations benzalkonium chloride is used as the preservative, thus accumulation in the accessory structures of eye e.g. accumulation of timolol maleate or benzalkonium chloride in the conjunctiva could occur (Liu *et al.*, 2004). When determining an acute exposure period that would be representative of such an *in vivo* system, careful consideration needs to be made as to the associated structures of the eye and cornea which afford some protection or enhancement of the irritating effect of exogenous chemicals. *In vivo* the cornea's first line of defence against chemical insult, apart from the upper and lower palpebrae (Prabhasawat and Tseng, 1998), is the tear film. Chemical irritants dissolved in a solution representative of the tear fluid proteins have less affect on cell viability, than when dissolved in phosphate buffered solution alone (Cheng *et al.*, 1995), highlighting the importance of the tear film as a defence against chemical insult. A J-HCET monolayer is more susceptible to irritant exposure than either a stratified skin model (Ward *et al.*, 1998) or the cornea *in vivo*. It has been suggested that these defensive systems clear 80% of the exogenous chemical from the ocular surface within 60 seconds of the initial exposure (Shaw *et al.*, 1990). The J-HCET monolayer model, used in this study does, not possess these defence mechanisms but nevertheless, an acute exposure time must be selected that is representative of the *in vivo* system, (Shaw *et al.*, 1990) and thus 60 seconds is chosen, with thorough post washing to mimic tearing and blinking. The washing of

the test material is vital especially with viscous material such as T20 as it has been demonstrated (Ward *et al.*, 1997b) that ineffective washing can lead to a longer exposure period than intended.

### **6.3.3 Effects of chronic surfactant exposure on J-HCET cultures**

It was probable that the subcytotoxic concentration of surfactants required for continued viability over a 21 day period will fall well below that of the CMC as above this level, surfactants tend to emulsify phospholipids bilayers (Rhein *et al.*, 1990). Rhein *et al.*, 1990; McFadden, 2001 suggested that, at lower concentrations, below the CMC, surfactants tend to be incorporate into the plasma membrane and bind to integral membrane proteins, including adhesion molecules. The concentrations of surfactants in growth medium that would allow sustained J-HCET viability over a 21 day period, ranked as T20, SDS, CAPB and BAK, from highest to lowest there being a 10,000 time difference between the non-ionic T20 and the cationic BAK. The medium supplement employed (HKGS or HKGS-V2) did not affect this response. Suggesting that the measured decreases in the ability of the J-HCET monolayers to internalise neutral red (Borenfreund and Puerner, 1985) was due to cellular loss, and not, alterations in plasma membrane transport mechanisms as a consequence of surfactant assimilation (Rhein *et al.*, 1990; McFadden, 2001). These results only concern the viability in terms of neutral red uptake and protein content of a static (none or limited replicative ability) J-HCET monolayer. The critical issue was the effects that these subcytotoxic surfactant concentrations had not only on the growth rate, but also barrier integrity and redox potential of J-HCET cells over a 21 day exposure period.

Previous measurements of the population doubling time have demonstrated that the supplement employed (HKGS or HKGS-V2) can have a significant effect on the growth rate of the J-HCET cell line if the coating matrix was omitted with the HKGS-V2 supplements. The population doubling time of J-HCET cultured in medium containing HKGS supplements or HKGS-V2 supplements without the use of the recommended attachment factor was unaffected by chronic SDS;  $4\mu\text{gml}^{-1}$ , T20;  $25\mu\text{gml}^{-1}$ , CAPB;  $3\mu\text{gml}^{-1}$  or BAK;  $0.0025\mu\text{gml}^{-1}$  exposure. However, when the J-HCET population doubling time was determined for J-HCET cultured in medium containing HKGS-V2 supplements in the presence of the attachment matrix, it was observed that the growth rates were comparable to those expected if the attachment matrix was omitted. Thus the surfactants appear to affect or degrade the matrix. Whilst the matrix coating was not required when the cells are grown on the inserts, and hence the chronic exposure may not disrupt differentially the cells grown in the HKGS-V2, the potential effects on direct culturing on a 96 well plates does need to be taken into account.

The resazurin reduction assay has been widely used to assess bacterial or yeast contamination in biological fluids and milk (Erb and Ehlers, 1950), to measure the viability of sperm by colorimetry (O'Brien *et al.*, 2000) for cytotoxicity studies, in the form of Alamar Blue<sup>TM</sup>, and the effects of drugs on specific mammalian cell lines (Page *et al.*, 1993; Suggs *et al.*, 1999; Lee *et al.*, 2000). The assay has been compared to other viability assays and demonstrated a good correlation in predicting cytotoxicity, e.g. with the trypan blue exclusion-(Gazzano-Santoro *et al.*, 1997), and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide salt (MTT) assay

(Black *et al.*, 1999) whilst allowing for repeated or continuous measurements (Goegan *et al.*, 1994; Vian *et al.*, 1994; Clothier and Sansom, 1996; O'Brien *et al.*, 2000). This cannot be achieved with assays that require fixation or destruction of the cells to determine the endpoint e.g. the MTT, NRU, NRR and total protein assays. Chronic surfactant exposure was not associated with a significant change in the ability of J-HCET monolayers to convert resazurin to resorufin when compared with unexposed cultures. This suggests that chronic surfactant exposure at the concentrations investigated (SDS:  $4\mu\text{gml}^{-1}$ ; BAK:  $0.0025\mu\text{gml}^{-1}$ ; T20:  $25\mu\text{gml}^{-1}$  or CAPB:  $2.99\mu\text{gml}^{-1}$ ) do not significantly interfere with the J-HCET respiratory pathways involved with resazurin reduction.

#### **6.3.4 Effect of chronic exposure on J-HCET monolayer barrier function**

Ward *et al.*, (1997a) reported that a cationic surfactants i.e. benzalkonium chloride appeared to adsorb to particular types of membrane material and cause sodium fluorescein to bind to it on the membrane producing erroneous leakage results, and a yellow insert. Ward *et al.*, (1997a) did not evaluate polycarbonate inserts and with no literature information on benzalkonium chloride interacting with polycarbonate membranes, preliminary studies were run that demonstrated that exposing the no-cell insert to this surfactant did not result in erroneous leakage data. Utilising the combined fluorescein leakage- resazurin reduction assay to measure the permeability and activity of the J-HCET monolayer the subcytotoxic surfactant (SDS;  $4\mu\text{gml}^{-1}$ , T20;  $25\mu\text{gml}^{-1}$ , CAPB  $2.99\mu\text{gml}^{-1}$  or BAK;  $0.0025\mu\text{gml}^{-1}$ ) exposure had no detectable effects on barrier function or cell activity over the whole 21 day exposure period regardless of supplement (HKGS or HKGS-V2) employed. Morphological



analysis of the essential components of the tight junction (f-actin, ZO-1 and occludin) revealed that between days 19 and 21 of continuous BAK exposure the localisation of ZO-1 to the membrane of abutting J-HCET is significantly disrupted even in the presence of 1000 $\mu$ M calcium. This did not result in an increase in fluorescein leakage. This does not preclude the possibility that a reaction to a subsequent higher acute exposure would not modulate the barrier function. It is possible that detoxification or repair pathways had been augmented by the chronic exposure, or that the ZO-1 associated with non TJ's e.g. adherens junctions, was what was affected. This requires further investigation.

A number of variants of the fluorescein leakage assay exist. A prevalidation trial of one standardised method has recently been completed (Southee and Curren, 1997). Sadly this was not successful in that the Protocol was not sufficiently defined and those conducting the assay did not follow the protocol or meet to discuss and resolve protocol problems. To improve the predictive capacity of the fluorescein leakage assay the resazurin assay was added. The rationale for this was that it may help to distinguish between those chemicals that cause significant fluorescein leakage via damaging all the adhesion molecules but not killing cells from those which kill a few cells but do not affect adhesion molecules expression significantly. Both could result in say 60% leakage with a no cell insert giving 100% and a confluent layer 6 %. In the former case, where adhesion molecules alone were affected, recovery could be rapid since upon the removal of the surfactant the cell adhesion molecules could be re-expressed. In the later case cell division would be required to restore the cell numbers and hence would take over 24 hours. It was upon this basis that this kind of approach was taken to predict mild from more aggressive ingredients and

formulations for cosmetic use (Hubbard *et al.*, 1994) and for predicting human skin patch test data for hand wash solutions (Clothier *et al.*, 2002). It was determined that, in correlation with the present study, *in vitro*, T20 was less damaging than SDS which was less damaging than CAPB and BAK as was also noted in the Draize scores (1944) from historical data (ECETOC, 1992). The MMAS scores (ECETOC, 1998) are 4, 16, and 56.3 respectively with concentration of 100%, 3%, 1% for T20, SLS and BAK.

When compared with the model developed during the current study, results of the same order of magnitude were observed and thus the same irritancy potential would be predicted. The concentration of chemicals required to damage this monolayer system were greater, possibly as a consequence of the shorter exposure period (60 seconds). Hence, the prediction of ocular irritancy potential from this combined *in vitro* assay system is based not only on the immediate effects but, as for the Draize score, also on the rate of recovery of cell-junction integrity and cell viability. The recovery profiles demonstrated that at high concentrations of T20 the change in resazurin reduction capacity of the cells was directly related to the change in cell-junction integrity. A distinction between the effects on tight junctions and resazurin reduction was observed at the highest BAK concentration. Fluorescein leakage was increased but no significant modulation of resazurin reduction occurred. Therefore, impairment of generalised membrane function would be required to result in a decrease in resazurin reduction and overall viability (Larson *et al.*, 1997). Recovery occurred with all test materials, in the present study, at concentrations that only depress both the resazurin reduction and barrier function integrity. At the highest concentration of T20, extensive damage to the monolayer was observed along with a

significant loss of ability to recovery. This was in correlation with the study of Clothier *et al.*, (1999) who reported extensive damage to the MDCK monolayer was associated with a significant loss in the ability of the cells to recover over the 96 hour period. In addition, although the irritancy potential of the test materials was of the same order as when J-HCET were cultured on tissue culture plasticware, the concentration required to achieve a similar decrease in resazurin reduction on a polycarbonate membrane was higher. This confirms the hypothesis of Ward *et al.*, (1997a, 1998) that culture conditions significantly affect the irritancy potential of chemical concentrations *in vitro*.

As reported (Hubbard *et al.*, 1994) the initial effects and the rate of recovery combined allow for discrimination between irritancy potential of chemicals and concentrations of the same test agent. Results concerning the effects on monolayers cultured in either HKGS or HKGS-V2 supplemented medium were comparable i.e. HKGS-V2 supplements do not modulate the toxicity of surfactants to molecules of the tight junction.

### **6.3.5 Chronic and acute surfactant exposure**

Numerous methods are available for the determination of *in vitro* cytotoxicity resulting from a single acute exposure to a chemical (Clothier *et al.*, 1997; Eskes and Zuang, 2005). Evaluation of toxic insult to confluent layers of cells using high concentrations combined with short exposure times e.g. Neutral Red Release assay (Reader *et al.*, 1990), through to low concentrations with longer exposure during exponential cell growth (e.g. the Kenacid Blue assay; Knox *et al.*, 1986) are in

routine use. In the MEIC scheme a whole range of acute assay methods are reviewed and compared (Clemedson *et al.*, 1996). However, many chemicals present significant toxicity *in vivo* on repeat exposure and hence methods are being developed *in vitro* to facilitate the evaluation of effects following repeat exposure (Clothier *et al.*, 1997). *In vitro* chronic exposure assays need to be designed with the ability to monitor changing responses during the exposure period. Techniques should be adaptable, since the mechanisms of toxicity could change with the dose, cell target and number of exposures. Assays could adopt different treatment regimens, for example ranging from daily re-exposure for a fixed time, as is adopted *in vivo* with human patch testing of cosmetics, to repeat exposure once recovery has occurred from the previous insult as described for the evaluation of repeat exposure to mild surfactants (Clothier and Samson, 1996). In this study, two different approaches were employed. Initially, cells were seeded and specific parameters were measured (resazurin reduction, neutral red uptake, population doubling time, total protein content and fluorescein leakage) during continuous exposure to subcytotoxic concentrations of the test materials for 21 days. Secondly, J-HCET were cultured in medium containing the test materials and subsequently seeded out to determine the effects that the chronic exposure had upon the acute toxicity of the test materials. Previous acute exposures to these chemicals gave a ranking of: T20, SDS, CAPB and BAK in increasing order of toxicity as observed with human keratinocytes (Ward *et al.*, 1998).

However, when J-HCET were continuously exposed to concentrations, that are below the detectable cytotoxic range, a sub-acute assay, result in cumulative effects that alter the toxicity and recovery profiles to a subsequent single acute challenge

(Clothier *et al.*, 1999). An analysis of the parameters measured during these studies reveal that the ratio of both neutral red uptake and resorufin production to protein content are constant regardless of chronic – acute exposure history. This suggests that the modulation of toxic response as a consequence of previous exposure history is related to cell loss from the culture surface and not perturbations in cellular metabolism. However, the precise mechanism involved is unclear. This illustrates that while such an approach can be used to evaluate chronic toxicity, more extensive assay methods to identify the precise changes that have occurred within the cells are required. Not only are enzymes and receptor assays required but also assays examining specific detoxification pathways which allows for the repeat assaying of the same cell population (Clothier and Samson, 1996). The advent of 3T3 cells with specific cytochrome P-450 enzyme capacities inserted opens a further facet to *in vitro* models to examine chronic chemical toxicity resulting from specific metabolism (DeGroene *et al.*, 1995). Bull *et al.*, (1999) showed that the various pathways of Imipramine metabolism can be investigated using this approach. Extending the use of use of such cell lines to chronic/repeat evaluation was beyond the scope of this project.

#### **6.4 Prolonged use of timolol maleate in the treatment of glaucoma**

The reduction-oxidation (redox) state of the cell is a consequence of the balance between the levels of oxidising reactive oxygen species (ROS) and reducing equivalents. Although the total antioxidant status of cells may appear sufficient to protect them from oxidative stress, the necessary defence mechanisms may not be sufficiently functional. Particularly large numbers of a certain radical species, and

the type of antioxidants needed to deal with these species where in short supply, excess ROS were free to strip electrons from molecules in cell walls and genetic material and also to oxidise dietary fats in the blood, a chain reaction yielding more free radicals and, therefore, more extensive oxidative stress would occur. Glutathione is the principle non-protein thiol involved in the antioxidant cellular defence (Pastore *et al.*, 2003). Glutathione is a ubiquitous molecule that is produced in all organs, especially the liver. Intracellularly, it is found in millimolar concentrations and the level of glutathione is much greater than that of cysteine and seems to serve as a storage form of cysteine moieties (Pastore *et al.*, 2003). In cells, total glutathione can be free or bound to proteins, free glutathione is present mainly in its reduced form. This may be converted to the oxidised form during oxidative stress, and can be reverted to the reduced form by the action of glutathione reductase (Pastore *et al.*, 2003). Glutathione provides reducing capacity for several reactions, for example, the formation of deoxyribonucleotides by ribonucleotide reductase (Pastore *et al.*, 2003) and the reduction of dehydroascorbate to ascorbate. Glutathione functions in the detoxification of hydrogen peroxide and free radicals. It also plays a role in the detoxification of a variety of xenobiotics, which interact with glutathione and are ultimately excreted (Pastore *et al.*, 2003). The synthesis and degradation of glutathione takes place by reactions of the  $\gamma$ -glutamyl cycle. Since this cycle leads to the synthesis of glutathione, its function is closely connected with the several metabolic and physiological functions that are performed by this ubiquitous tripeptide (Klatt and Lamas 2000; Pastore *et al.*, 2003). When an inhibitor of glutathione synthesis, such as buthionine sulfoxamine (BSO), is given, there is a decrease in the intracellular levels of glutathione (Pastore *et al.*, 2003). Even a complete block does not stop the function of the  $\gamma$ -glutamyl cycle, since



glutamate may be produced by other pathways, such as hydrolysis of glutamine and transamination or reductive amination of  $\alpha$ -ketoglutarate (Griffith and Meister, 1979). It has been established that an increase in the glutathione level of a cell makes it more resistant to certain xenobiotics, whilst exposures that reduce cellular glutathione levels usually promotes sensitivity to certain drugs, radiation and oxygen (Klatt and Lamas, 2000 Pastore *et al.*, 2003). The prolonged use of timolol maleate as a treatment for glaucoma has been associated with discontinuation of treatment as a result of ocular irritation (Uusitalo *et al.*, 1999; Aggarwal and Kaur, 2005). Since timolol maleate is detoxified *in vivo* via conjugation with glutathione (GSH) (Megaw, 1984; Riley, 1990) a proposed mechanism for the irritancy associated with long term use may be alterations in the  $\gamma$ -glutamyl cycle resulting in a net decrease in intracellular GSH available for timolol detoxification. This would result in an increased sensitivity to oxidising chemical insult to the corneal epithelium as redox status depends upon the relative amounts of the reduced and oxidised forms of glutathione (GSH/GSSH). J-HCET cultures exposed to BSO *in vitro* confirm that the toxicity of timolol to this human corneal cells line was enhanced by suppressing the activity of  $\gamma$ -glutamylcysteine synthetase (Griffith and Meister, 1979) through irreversible inhibition resulting in a decrease in intracellular GSH levels. This clearly demonstrates that the J-HCET model may be employed to monitor detoxification pathways, specifically conjugation to GSH. It would be possible, in the future to use cell tracker green (Clothier *et al.*, submitted for publication) to also visualise the detoxification through conjugation to GSH as has been recently examined with the photoproduct of Bithionol in this lab (Reid *et al.*, submitted for publication).

It has been suggested (Sponsel *et al.*, 1999) that timolol maleate accumulates in the conjunctival cul-de-sac by both capillary and gravitational force, thereby, remaining in sustained intimate contact with the ocular surface at concentrations significantly lower than the original exposure. Utilising the J-HCET model, a timolol maleate concentration was identified that did not affect the viability, population doubling time or intracellular GSH levels over a 21 day incubation period. Although long term use of timolol containing pharmaceuticals have been reported to be associated with irritancy, a single exposure to clinically relevant concentrations ( $2.5\text{mgml}^{-1}$ ) has not been associated with aggravation of the ocular surface (Megaw, 1984; Riley, 1990; Pastore *et al.*, 2003). The finding that *in vitro* acute exposures to levels below that found in pharmaceutical preparations may result in higher intracellular GSH concentrations. This is due to upregulation of the  $\gamma$ -glutamyl cycle in order to detoxify timolol maleate, a xenobiotic conjugated to GSH for its removal from the cell (Griffith and Meister, 1979; Pastore *et al.*, 2003). The specific enzyme pathway responsible for the upregulation of GSH is almost certainly the  $\gamma$ -glutamylcysteine synthetase pathway as when cells are cultured in the presence of BSO and subsequently exposed to timolol, the upregulation of GSH was no longer observed. A similar effect was observed in J-HCET cultured in subcytotoxic timolol concentrations for 21 days and subsequently exposed to an acute level. Although the subcytotoxic concentration determined demonstrates no overt toxicity with regard to viability or GSH concentration, upon a acute exposure, it was found that the toxicity of timolol was significantly increased. This suggests that the  $\gamma$ -glutamyl cycle cannot upregulate GSH levels over a sustained period upon acute exposures as a consequence of chronic pre-exposure.

#### **6.4.1 Timolol exposure and barrier function**

Measurements reveal that an acute timolol exposure significantly increased the leakage of fluorescein across the monolayer with no decrease in viability or protein content. In addition, morphological analysis reveal significant alterations in the localisation of both ZO-1 and occludin immediately following exposure suggesting that the increase in leakage was due to disruption of tight junction integrity. It had been demonstrated that the expression and localisation of the components of the tight junction was dependent upon the correct calcium concentration (Wilkinson *et al.*, 2005). Timolol maleate is known to influence cellular sodium and calcium channels *in vivo* (Aggarwal and Kaur, 2005) and these results suggest timolol maleate may modulate tight junctions *in vitro*. Complete recovery of the barrier function was observed in unexposed monolayers; however, those pre-exposed to timolol only demonstrated partial recovery. This suggests that the recovery of barrier function may be modulated by previous exposure history.

#### **6.5 Conclusion**

This study has shown that the development of such a reliable and reproducible *in vitro* assay for chronic/repeat ocular irritation it is vital. The mechanistic relevance of the endpoints chosen and monolayer or multilayer ultrastructure is considered to be a vital component. The development of cell based *in vitro* systems to predict human responses to chronic/repeat ocular irritation is required (Balls and Worth, 2002). This assay does address the components of the validation process and not “invalidation” as outlined by Balls and Combes (2005).

## **6.6 Future Work**

### **6.6.1 Stratification of the J-HCET model**

In addition to provide a species specific model it is desirable that an *in vitro* model afford the potential to express mechanistically relevant responses that can be used for predicting human ocular toxicity and recovery potential. Although the model developed during the present study employs human corneal epithelial cells, the stratification observed *in vivo* is not present. In the future it may be possible to develop techniques which will enable the stratification of the J-HCET model to more accurately represent the *in vivo* structure of the human cornea.

### **6.6.2 Monitor tight junction integrity using TEM**

Ward *et al.*, (1997b) employed TEM to visualise the effects that surfactant challenge had upon the ultrastructure of the Gillette corneal cell line. This technique could be adapted to visualise any subtle alterations in ultrastructure as a consequence of chronic exposure, and subsequently following an acute exposure to the test surfactants and timolol maleate.

### **6.6.3 Effects of benzalkonium chloride on timolol toxicity *in vitro***

Ophthalmic liquid formulations such as timolol contain penetration enhancers and antimicrobial agents (Chetoni *et al.*, 2003) e.g. BAK, which enhances trans-corneal drug delivery and suppresses bacterial growth. Chetoni *et al.*, (2003) suggested that

the long term irritancy of timolol maleate is due to the presence of penetration enhancers such as benzalkonium chloride as part of the formulation. It has been demonstrated during the current study that prolonged exposure to benzalkonium chloride significantly alters the expression of ZO-1 *in vitro*, without altering the flux of fluorescein or viability. In addition, the toxicity of benzalkonium chloride has been demonstrated to be modulated by previous exposure history. In the future, studies may be conducted concerning the effects that benzalkonium chloride pre-exposure has upon the toxicity of timolol, and that of formulations of timolol containing benzalkonium chloride as a preservative. This may lead to a further understanding of the mechanisms involved in the irritancy of timolol over prolonged use.

#### **6.6.4 Visualisation of GSH conjugation to xenobiotics *in vitro***

It has been demonstrated that intracellular levels of GSH may be measured *in vitro* utilising a simple colorimetric assay, and that the GSH levels may be modulated by xenobiotics e.g. timolol maleate and BSO. In addition to quantifying the intracellular GSH it would be possible in the future to use cell tracker green to visualise the cytoplasmic distribution of GSH and monitor xenobiotic detoxification through conjugation to GSH, as has been recently examined with the photoproduct of Bithionol in this laboratory (Reid *et al.*, submitted for publication).

## **Chapter 7**

### ***References***



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